ABSTRACT

Title of Document: NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*: PRESENCE IN FOOD, PATHOGENICITY ISLAND AND MOLECULAR EVOLUTION

Wenting Ju, Doctor of Philosophy, 2013

Directed By: Professor, Jianghong Meng
Department of Nutrition and Food Science

Non-O157 Shiga toxin-producing *Escherichia coli* **(**STEC) are emerging foodborne pathogens that can cause life-threatening hemolytic uremic syndrome (HUS) as well as foodborne outbreaks worldwide. Compared with O157, non-O157 STEC are not well studied. The objective of this study is to determine the presence of non-O157 STEC in food, identity virulence markers to distinguish highly pathogenic strains and determine its phylogenetic relationship.

Ground beef (259) and pork (231) samples were collected weekly for one year period (2009-2010) to determine the prevalence of non-O157 in retail meat in the Washington, D.C., area. Colony hybridization was used to identify potential non-O157 STEC after PCR screening for the *stx* gene from the enrichment broth. Non-O157 isolates were characterized phenotypically and genotypically by serogrouping, virulence genes, pulsed-field gel electrophoresis (PFGE), cytotoxicity assay and

antimicrobial susceptibility assay. The results demonstrated that both ground beef and pork were contaminated with heterogeneous non-O157 STEC at similar levels (5%) and a subset of isolates were potential human pathogens based on the virulence genes and serogrouping information. In addition, this study demonstrated that antimicrobial resistance was common in STEC isolates from retail meat.

Additionally, the distribution of pathogenicity islands (OI-122, OI-57, OI-43/48 and high pathogenicity island) was investigated in 98 STEC strains classified into 5 seropathotypes. PCR-RFLP was used to determine *eae* and *stx* subtypes, and 14 PCR assays were used to amplify virulence marker genes of PAIs. In addition, phylogenetic dendrograms were constructed for *pagC* and *iha*. The prevalence of OI-122 and OI-57 was significantly higher in seropathotypes associated with severe diseases and outbreaks than in other seropathotypes ($P<0.0001$). Most virulence genes located on OI-122, OI-43/48 and OI-57 were found more often in seropathotypes associated with severe disease and outbreak than in other seropathotypes ($P<0.0001$). Interestingly, OI-122, OI-57 and OI-43/48 were found highly associated with *eae*-positive STEC strains, while the presence of HPI mostly occurred independently of *eae* presence.

Last, the phylogenetic relationship of non-O157 STEC was determined based on whole genome wide study of 33 STEC and 10 other pathogenic *E. coli*. Dendrogram of PFGE, MLST and whole genome level single nucleotide polymorphisms indicated that O26:H11 and O111:H11 were closely related and may have a common ancestor.

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By

Wenting Ju

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Advisory Committee:
Professor Jianghong Meng, Chair
Associate Professor Kevin McIver
Assistant Professor Qin Wang
Dr. Mickey Parish
Dr. Jie Zheng

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Table of Contents

Acknowledgements ii
List of Tables iv
List of Figures v
List of Abbreviations vi
CHAPTER I: LITERATURE REVIEW 1
Shiga toxin-producing *E. coli*: an overview 1
Serotyping and seropathotypes 2
Non-O157 STEC: underrated pathogens 4
Outbreak caused by non-O157 STEC 5
Virulence factors 6
Reservoirs and transmission 11
Contamination in meat 12
Isolation and detection challenge 12
Project overview 15
References 17
CHAPTER II: NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN RETAIL GROUND BEEF AND PORK IN THEWASHINGTON D.C. AREA 28
Abstract 28
Introduction 29
Materials and Methods 31
Results 35
Discussion 38
References 43
CHAPTER III: DISTRIBUTION OF PATHOGENICITY ISLANDS OI-122, OI-43/48, OI-57 AND HIGH PATHOGENICITY ISLAND (HPI) IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* 58
Abstract 58
Introduction 59
Materials and Methods 62
Results 65
Discussion 69
References 75
CHAPTER IV: PHYLOGENETIC ANALYSIS OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* BY WHOLE GENOME SEQUENCING 93
Abstract 93
Introduction 94
Materials and Methods 95
Results 97
Discussion 99
References 102
CHAPTER V: SUMMARY AND FUTURE STUDY 112
MASTER REFERENCES 117

List of Tables

TABLE II-1. Oligonucleotide primers based on wzy for PCR serogrouping..... 52

TABLE II-2. Antimicrobial resistance profiles of non-O157 STEC recovered from retail ground beef and pork. 53

TABLE II1-1. Distribution of *eae* subtypes, and virulence genes of OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) by serotypes..... 83

TABLE III-2. Distribution of pathogenicity islands (PAIs) in seropathotypes associated outbreak (SPT A and B) and severe disease (SPT A, B and C). 85

TABLE III-3. Distribution of virulence genes located at pathogenicity islands (PAIs) in seropathotypes associated outbreak (SPT A and B) and severe disease (SPT A, B and C). 86

TABLE III-4. Characterization and presence of PAIs associated virulence genes among O157:H7 and O157:NM..... 87

TABLE IV-1. Serotypes, pathotypes, toxin genotypes, sources and genome sizes of *Escherichia coli* strains used in this study. 106

TABLE IV-2. Pairwise distance matrix analysis of six selected groups. 108

List of Figures

FIGII-1. Dendrogram of PFGE profiles with XbaI of 32 STEC isolates from ground beef and pork..... 54

FIG II-2. Vero cell cytotoxicity of 32 STEC isolates from ground beef and pork. 56

FIG III-1. Distribution of OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) in STEC strains (n=98) of different seropathotype..... 88

FIG III-2. Distribution of virulence genes located on OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) between *eae*-positive (n=54) and *eae*-negative STEC (n=44) strains. 89

FIG III-3. Phylogenetic tree based on *iha* sequences from 67 *E. coli* and *Shigella* strains. 90

FIG III-4. Phylogenetic tree based on *pagC* sequences from 35 pathogenic *E. coli* strains. 92

Figure IV-1. Dendrogram of PFGE profiles of 15 O26, O103 and O111 STEC isolates..... 109

Figure IV-2. Dendrogram of MLST analyses using *aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*. 110

Figure IV-3. Parsimony phylogenetic tree of 43 *E. coli* from diverse pathotypes based on genome wide single nucleotide polymorphisms (SNPs) with 10,000 iterations. 111

List of Abbreviations

AidA	Adhesin involved in diffuse adherence
ATCC	American Type Culture Collection
BioNJ	Bio Neighbor Joining
CDC	Centers for Disease Control and Prevention
CVM	Center for Veterinary Medicine
DAEC	Diffuse-adherent *E. coli*
DEC	Diarrheagenic *E. coli*
EAEC	Enteroaggregative *E. coli*
Efa	Enterohemorrhagic *E. coli* factor for adherence
EHEC	Enterohemorrhagic *E. coli*
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *E. coli*
EMEM	Eagle's Minimum Essential Medium
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FDA	Food and Drug Administration
Gb3	Globotriaosylceramide
Gb4	Gloctetraosylceramide
GUD	Glucuronidase
HC	Hemorrhagic colitis
HlyA	Hemolysin A
HPI	High pathogenicity island

HUS	Hemolytic uremic syndrome
Iha	Iron-regulated gene A
LEE	Locus of enterocyte effacement
LifA	Lymphostatin
LPF	Long polar fimbriae
ML	Maximum likelihood
MLST	Multi-locus sequence typing
MLVA	Multiple locus variable-number tandem repeat analysis
MRA	Molecular risk assessment
Nle	Non-LEE effector
NMEC	Neonatal meningitis *E. coli*
PAI	Pathogenicity island
PagC	*phoP*-activated gene C
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAJ	Recto-anal conjunction
RTX	Repeats in toxin
Saa	STEC autoagglutinating adhesion
SNPs	Single nucleotide polymorphisms
SPT	Seropathotype
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin
TTSS	Type III secretion system

TNT	Tree analysis using New Technology
UPEC	Uropathogenic *E. coli*
UPGMA	Unweighted pair group means with arithmetic averages
VT	Vero toxin
VTEC	Verocytotoxin-producing *E. coli*
WGS	Whole genome sequencing

CHAPTER I: LITERATURE REVIEW

E. coli are Gram negative, short rod, and facultative anaerobic bacteria (1). Optimal growth of *E. coli* could be reached at 37 ℃, but it also can grow well at 42 ℃ (1). The main niches of *E. coli* are the intestinal tract of humans and warm-blooded animals, where they appear almost ubiquitously. Most *E. coli* are commensal bacteria and do not cause any disease except to immunocompromised people. However, during evolution, certain subsets of *E. coli* acquire a combination of virulence genes and become human pathogenic strains. Until now, six main pathotypes of diarrheagenic *E. coli* have been identified based on their virulence genes, pathogenic mechanisms and clinical symptoms. The six pathotypes associated with disease are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse-adherent *E. coli* (DAEC). Recently, an emerging pathotype called Shiga toxin-producing EAEC has been identified that harbors a combination of virulence factors from STEC and EAEC. Of all pathogenic *E. coli*, STEC are the most devastating and a major public health concern for its association with large foodborne outbreaks and life-threatening hemolytic uremic syndrome (HUS) (2).

Shiga toxin-producing *E. coli*: an overview

Shiga toxin-producing *E. coli* (STEC) are *E. coli* producing one or more Shiga toxin (s). STEC can also be called Verocytotoxin-producing *E. coli* (VTEC) for its toxigenic effect to Vero cells (3). Generally speaking, STEC is primarily used in the United States, whereas VTEC is mostly used in Europe (2). STEC can cause a variety

of diseases such as watery diarrhea, bloody diarrhea, hemorrhagic colitis (HC), and HUS. Most of the time, STEC infections are self-limiting; however 5-10% of diarrhea infection develop into a potential life-threatening HUS (http://www.cdc.gov/ecoli/general/index.html/). The STEC causing HC and HUS are also called enterohemorrhagic *E. coli* (EHEC) (4). All EHEC are pathogenic, whereas not all STEC are pathogenic to human beings. *E. coli* O157:H7 are the most well-known serotype of STEC because of its strong association with outbreaks and HUS. However, non-O157 STEC can also cause human infection and some serotypes, such as O26, are considered as virulent as O157. In total, more than 470 serotypes of STEC have been isolated from humans; yet, less than 10 groups are responsible for the majority of cases (1). For example, in the United States, six of the most common serogroups (O157, O26, O111, O121, O45 and O145) made up 82% of non-O157 human isolates in 2009 (5).

Serotyping and seropathotypes

Serotyping is one of the most extensive methods used to characterize *E. coli*. Serotyping is based on the immunologic reactivity of two surface structures: cell wall polysaccharide (LPS) (O antigen) and flagella (H antigen). O antigen determines serogroup of a strain, and H antigen identifies its serotype. In total, there are 174 O antigens (numbered from 1 to 181, with 31, 47, 67, 72, 93, 94, and 122 deleted) and 53 H antigens that have been identified with various O and H combinations (1).

Karmali classified all STEC into five seropathotypes based on a specific serotype's association with diarrhea, HUS, and outbreak (6). Seropathotype A (SPT A) consists of O157:H7 and O157:NM, which are considered to be most virulent

STEC and they are commonly associated with foodborne outbreak and severe disease. Seropathotype B (SPT B) consists of O26:H11, O103:H2, O111:H8/NM, O121:H19, and O145:NM. Strains in SPT B can cause severe disease and outbreaks similar to SPT A, but occur at lower frequency. Seropathotype C (SPT C) is composed of serotypes that are infrequently implicated in sporadic HUS and rarely associated with outbreaks such as O91:H21, O113:H21, O45:H2. Seropathotype D (SPT D) is composed of numerous serotypes that have been implicated in sporadic cases of diarrhea, but never in HC and HUS. Seropathotype E (SPT E) is composed of the many STEC serotypes that are mainly isolated from animal and food products and have not been implicated in human disease (1).

Although serotyping is often regarded as the starting point of STEC characterization, there are many disadvantages of this technique. First of all, the serotyping process is tedious and lab intensive. Second, cross-reaction also happens between different serotypes and gives confusing results. Third, it is common that O and H antigens cannot be serotyped because they are not within the international scheme. Last, only a small number of labs worldwide are authorized for *E. coli* serotyping, which makes it not accessible for some researchers.

Although serotyping can be used to reveal the diversity of STEC, it is often not effective enough to distinguish STEC strains. Thus, subtyping methods beyond the level of serotyping are needed for epidemiological study, outbreak investigation and early detection of geographical distribution of foodborne pathogens. A variety of subtyping methods such as pulsed-field gel electrophoresis (PFGE), multi-locus

sequence typing (MLST), multiple locus variable-number tandem repeat analysis (MLVA) and phage typing, have been developed to differentiate STEC.

Non-O157 STEC: underrated pathogens

It is well known that O157 causes most of the STEC HUS infections and foodborne outbreaks worldwide. However, more and more data show that non-O157 STEC are as important as O157 and illnesses caused by non-O157 are rising worldwide (7). In the United States, the active surveillance of non-O157 began in 2001. Since then, the number of cases caused by non-O157 is increasing sharply. According to recent surveillance by the Center for Disease Control and Prevention (CDC), non-O157 can cause almost twice as much infection as O157:H7. Specifically, O157:H7 and O157:NM cause about 63,153 cases of diseases annually and non-O57 STEC can cause 112,752 cases each year (8). In the Netherlands, survey data has shown that 80% of STEC infections are caused by non-O157 (9). Similarly, 74% of STEC infections are non-O157 in Denmark (10). However, the real number of diseases caused by non-O157 STEC may still be underestimated. Currently, there are only a few public health clinical labs routinely looking for them (11). In addition, the isolation of non-O157 is time consuming and lab intensive, and there is no biochemical maker that can distinguish non-O157 from commensal *E. coli*, which makes isolation of non-O157 a big challenge. An enhanced surveillance targeting STEC by comprehensive laboratory analysis that reported that non-O157 STEC increased from < 1 to 11 cases/year/100,000 populations, supported that non-O157 is still underreported.

Outbreak caused by non-O157 STEC

The first recorded outbreak caused by non-O157 STEC occurred in Japan in 1984, and O145:H- was determined to be the causing serotype; however, the source of the outbreak was not identified (12). After this, outbreaks of non-O157 have been reported worldwide, including in the United States, Europe, Australia, and Japan. There have been more than 80 outbreaks worldwide associated with non-O157 since 1984 (13). The real number of outbreaks caused by non-O157 is likely more, because it is very hard to isolate and characterize non-O157 STEC from food and clinical samples.

In the United States, there were at least 24 outbreaks associated with non-O157 from 1990 to 2011 (5, 14). The first outbreak caused by non-O157 was reported in 1990 in Ohio. O111:NM was the serotype that caused a family outbreak, and the source of the outbreak was unknown (15). The largest non-O157 STEC outbreak in the United States was also caused by O111:NM; it occurred in Oklahoma in 2008. The outbreak led to 341 cases of illness: 26 patients developed HUS and 1 death occurred (16). Although the outbreak was traced back to a restaurant, an extensive effort has been made to identify the source, but no particular source has ever been identified (16).

Although some of the sources are not determined, the main transmission vehicles of non-O157 STEC associated outbreaks are food, water and person-to-person transmission. The food vehicles included milk, salad bars, beef, beef sausages, apple cider, lettuce, ice cream and sprouts (5). The serogroups associated were O111 (10), O121 (5), O26 (3), O45 (2), O104 (2), O103 (1) and O145 (1) (5).

Virulence factors

Shiga toxin

Shiga toxin is the primary virulence factor of STEC. Shiga toxin can be divided into two groups: Stx1 and Stx2. The Stx1 group is highly homogenous and consists of Stx1a, Stx1c and Stx1d (17, 18), whereas the Stx2 group contains several variants including Stx2a, Stx2b, Stx2c, Stx2d, Stx2dact, Stx2e, Stx2f and Stx2g (19-24). Although Stx1 and Stx2 share a common receptor and have the same intracellular mechanism of action, they only share 56% identity at the amino acid level (25). The Stx2 variants share about 84%-99% similarity to Stx2a (25). Shiga toxin types and an association with the clinical symptoms of STEC infection have been demonstrated (26). Stx2a, Stx2c and Stx2dact were demonstrated with high pathogenic STEC and the ability to cause HUS, whereas Stx1a, Stx1c, Stx2e and other Stx subtypes occurred mainly in patients with milder diarrhea, asymptomatic carriers or in animals (26).

Stxs are encoded by temperate lambdoid phages inserted at various positions within the STEC chromosomes (7). Phages are highly mobile genomic elements, and play an important role for horizontal gene transfer and diversification of bacteria genomes. Stxs are highly expressed when the lytic cycles of phage is induced (25). The production of Stx is regulated by several factors such as promoter activity, amplification of the copy number and toxin release (27). Once assembled, the final toxin secretion is achieved by lysis of the host cells.

Shiga toxins are AB_5 toxins. The A subunit (32 kDa) of the toxin possesses enzymatic activity that can cleave one adenine from the 28S rRNA, thus preventing

protein synthesis of the host cells, and leads to apoptosis. B pentamer (7.7 kDa per unit) binds to the receptor molecular globotriaosylceramide (Gb3) or gloctetraosylceramide (Gb4)(stx2e) located at the cell membrane (28). The A subunit and B subunit are linked through a disulfide bond. The toxin molecule is taken up into the cell through receptor-mediated endocytosis. Shiga toxin can be destroyed if the toxin carrying vesicle fuses with the lysosome. In susceptible cells, the toxin can be transported to the Golgi apparatus and endoplasmic reticulum, leading to cell apoptosis. In humans, STEC mainly colonizes the large intestine. Although the detailed mechanism is unknown, Shiga toxin can be transferred to the blood stream and disseminated to other organs that express Gb3. Binding sites in human tissue include kidney tubules, intestinal lymphoid aggregates, sinusoidal liver cells, alveolar macrophages, and peripheral blood leukocytes (29). Renal glomerular endothelium cells can express high levels of Gb3 in humans, which is the main reason that STEC infection can lead to kidney failure.

Intimin

Intimin, which is a 95 kDa outer membrane protein encoded by the *eae* gene, is associated with intimate attachment of STEC to intestinal epithelial cells (13). Two functional groups have been identified in Intimin: the highly conserved N terminus that inserts into the bacteria outer membrane and the C terminus that binds to translocated intimin receptor (Tir), integrin and nucleolin. Based on sequence and antigenic variation, at least 17 intimin types (α1, α2, β1, β2, γ1, γ2/ , δ/κ, ε, ζ, η, η2, ι, λ, μ, ν, ξ, ο) have been identified. The main difference between various intimin subtypes are sequences located at the C-terminus (30). STEC strains producing *stx2*

and *eae* genes are most associated with severe human illness. However, many STEC strains without *eae* have also been isolated from patients with severe disease.

Hemolysin

Hly is one member of the pore forming repeats in toxin (RTX) family toxins and that lyse erythrocytes, which can provide iron for bacteria from hemoglobin (2). Hly is encoded by four open reading frames (*hlyCADB*) and secreted into the extracellular space using a type I secretion system. Besides erythrocytes, Hly can cause membrane damage to a wide variety of other cell types and also may induce proinflammatory cytokine production (1). Hly presents in both *eae*-positive and *eae*-negative STEC. However, a Finish study showed that *hlyA* was positive in 92% of *eae*-positive strains, but only 35% in *eae*-negative strains (31).

Pathogenicity islands in STEC

Increasing evidence has shown that the main difference between pathogenic strains and non-pathogenic strains of the same or closely related species are virulence genes encoded on large, horizontally acquired "gene cassettes" referred to as pathogenicity islands (PAIs) (6). PAIs are genome regions that contain large blocks of virulence genes (32). There are several features shared by all pathogenicity islands. The size of PAIs is usually larger than 10 kb. The G+C content and codon usage of PAIs are usually different from the core genome. A t-RNA gene is often present in one side of PAIs. In addition, PAIs are often flanked by direct repeat sequences and its genome commonly contains mobile genetic elements, such as integrases, transposases and insertion sequences (32). PAIs can carry a variety of

virulence genes such as adhesion genes, type III secretion system (TTSS), invasion genes, enterotoxin, or siderphores.

In STEC, the locus of enterocyte effacement (LEE) is required for formation of attaching and effacing lesions and is the most characterized PAI (33). In EDL 933 O157:H7, LEE is 43 kb and carries five functional polycistronic gene clusters (LEE1-LEE5) consisting of 41 open reading frames. The five LEE functional modules include a type three secretion system; Intimin and its receptor; the secreted effectors EspF, EspG, EspZ, EspH, and CesT; the secreted translocators (EspA, EspD and EspB) that are required to inject effectors into host; and regulators such as Ler (LEE-encoded regulator), GrlA (global regulator of LEE) and GrlR (global regulator of LEE-repressor) (32).

Besides LEE, OI-O122 is another PAI that is well described (6, 34). In O157:H7, OI-122 is a 23 kb island. Several virulence genes have been identified in this island. Efa1 (EHEC factor for adherence) is an adhesin originally described in some EHEC strains (35). The *efa1* gene is almost identical to *lifA*, an EPEC gene encoding lymphostatin (LifA), which can inhibit the proliferation of mitogen-activated lymphocytes and the synthesis of proinflammatory cytokines (36). In addition, Efa1/LifA contributes to EPEC adherence to epithelial cells and is critical for intestinal colonization by *Citrobacter rodentium*, which is an AE lesion-producing bacterial pathogen of mice (37). PAI OI-122 also harbors genes that are very similar to *pagC* of *Salmonella enterica* serovar *Typhimurium*, *sen* of *Shigella flexneri*, and two *C. rodentium* non-LEE encoded effector genes (*nleB* and *nleE*). PagC is required for bacterial survival within macrophages and is immunogenic to humans, while *sen*

encodes an *S. flexneri* enterotoxin (6). NleB is linked to colonization and disease in mice (38). NleE induces polymorphonuclear (PMN) transepithelial migration and is involved in the blockage of NF- κB activation (39, 40).

OI-43 and OI-48 are duplicate genomic islands found in the EDL933 genome (41). OI-43/48 genes can be divided into three function groups. The first group is a gene cluster of seven genes (*ureDABCEFG*) related to urea resistance. The inactive aproprotein (Ure ABC) is activated by the addition of two Ni^{2+} ions to each of the three active sites in the enzyme. Accessory genes, Ure D, UreE, UreF and UreG, are all needed for the function of urease (42). The second group is a gene cluster for tellurite resistance. Ter^r in EHEC strain EDL933 is encoded by *ter*ZABCDEF (43). The third group includes putative adhesion genes *iha* (iron-regulated gene A) and *aidA-1* (Adhesin involved in diffuse adherence) (42).

OI-57 is another genomic island associated with virulence (44, 45). Three non-LEE secreted effectors (NleG2-3, NleG6-2 and NleG 5-2) are located in this island. Although the biological function of those three genes is not clear yet, similar proteins as NleG are related that act to compromise the immune response of the host.

The high pathogenicity island (HPI), which encodes for a siderophore (yersiniabactin) mediating iron-uptake system, was first detected in *Yersinia pestis* (46). HPI is required for full virulence expression in Yersinia. Interestingly, an orthologous and highly conserved HPI is widely distributed among different species and genera of the family *Enterobacteriaceae*. FyuA is an outer membrane protein acting as a receptor for ferric-yersiniabactin uptake and for the bacteriocin pesticin (47). Irp2 is involved in the process of yersiniabactin synthesis.

Reservoirs and transmission

STEC strains have been isolated from a variety of hosts such as pigs, sheep, goats, cats, deer, hogs, dogs, flies and birds (5). Ruminants, especially cattle, are recognized as the major reservoirs for STEC O157 and apparently also for the reservoirs of non-O157 STEC. In the United States, cattle are considered the major reservoirs for STEC. However, in Australia, sheep are the major carriers of STEC (1). There are more than 435 serotypes of STEC that have been isolated from cattle (1, 48). A German study found a high association with the population of cattle and the number of non-O157 infection cases (49). A large number of the serotypes isolated from animals has also been isolated from patients.

Cattle transit STEC to humans mainly through fecal shedding. Some cattle are "super shedders." Although they account for only a small portion of the whole cattle community, "super shedders" are responsible for over 95% of STEC shed (50). Intimate attachment of STEC in the recto-anal conjunction (RAJ) contributes to the high concentration of STEC in the feces and prolonged shedding (51).

STEC from cattle manure can be transferred to humans through contaminated meat, milk, and dairy products. Drinking water or lakes may also be contaminated by fecal materials. In addition, fruits and vegetables can be contaminated by irrigation water or directly by fertilized manure. Furthermore, non-O157 can be transmitted to humans by contact with animals or infected people within families, daycare centers or hospitals.

Contamination in meat

Non-O157 STEC has been isolated from a variety of foods such as raw milk, beef, minced meat, pork, cheese, sausage, ice cream and lettuce. For meat products, Sekala et al. reported a prevalence of 5.45% in beef in Canada (52). In France, Pradel reported that 3.9% of beef samples were contaminated with STEC (33). However, STEC was found in 12% of ground beef in Spain (53), and in 16% of ground beef in Australia (54). For pork products, very few studies are available for the contamination rates. Samapour et al. reported that 9 of 51 pork samples were positive for *stx* probe, but only one isolate was recovered (55). In Austria, Mayrhofer et al. reported 1.7% pork samples were positive for STEC in (56). In a recent study in Korea, only 2.0% of pork samples were positive for STEC (57).

Isolation and detection challenge

Sorbitol-MacConkey (SMAC) is the most widely used media to isolate O157 and is based on the fact that most of O157:H7 cannot ferment sorbitol. SMAC is an effective media to differentiate O157:H7 and inhibit the growth of Gram-positive bacteria by crystal violet and bile salt. However, it is almost impossible to differentiate non-O157 in SMAC because all non-O157 colonies will be pink to mauve on this media.

Currently, isolation of non-O157 STEC from food faces many challenges. Food samples usually contain a variety of high background flora and it is common that the target cells, non-O157 STEC cells, are present in low number (58). Food processing often injures the bacteria cells or transfers them into viable but nonculturable (VBNC) state. Other factors such as the complex food particles

involved and possible nonhomogeneous distribution of non-O157 STEC make recovery of them from food samples even harder (58). Enrichment of the food samples is often required to overcome these challenges. Enrichment can recover the injured and VBNC cells, dilute the effect of food matrix inhibitors, and increase the number of target cells by millions of times (58). Besides the common challenges facing isolation of all targets cells, there are some unique challenges for isolation of non-O157 STEC. STEC comprises over 400 serotypes and they differ greatly in their pathogenic potential and physical characteristics (11). There is no single biochemical characteristic that can distinguish non-O157 STEC from generic *E. coli*.

The most common media used for enriching non-O157 STEC are Trypticase Soy Broth (TSB), Buffered Peptone Water (BPW), and *E. coli* broth (EC). In order to inhibit the growth of Gram positive or other background flora, many researchers add bile salts and novobiocin to the base media (59). However, research has demonstrated that novobiocin can inhibit the growth of non-O157 and it is not recommended to add it to the media. Other antibiotics such as cefixime, vancomycin and selective agents (tellurite) are also used to inhibit background flora; however, some of non-O157 are sensitive to one or more of these agents and adding antibiotics to the media may affect recovery rate for non-O157 STEC from food (59). In addition to the enrichment media, the incubation temperature is very critical in recovering STEC from food samples. Several studies have found that enrichment at 42 ^{o}C is more effective for isolating non-O157 STEC than at 37 ^{o}C from food products such as ground beef, cheese, apple juice and radish sprouts (60-63). Baylis found that incubation temperature alone (37 ^{o}C versuss 42 ^{o}C) is not a significant

factor contributing to the growth of 20 STEC strains in pure culture (59). However, enrichment at 42 ^{o}C can effectively inhibit the growth of background flora (59). Recently, an acid treatment procedure was reported that can effectively reduce the background flora in fecal samples and enhance the recovery of non-O157 STEC (64). The newly released USDA non-O157 isolation protocol uses 42 ^{o}C incubation temperature and exposure to acid treatment to increase the STEC recovery rate (65).

PCR or real-time PCR (singular or multiplex) targeting *stx* genes post enrichment acts as an effective, rapid and very specific screening tool. However, the drawback of this method is that it may cause false positives because some other bacteria species such as *Shigella dysenteriae* also contains one or more *stx* genes. Not only other bacteria, but also free phage containing *stx* phage can cause a false positive. Enzyme immunoassay (EIA) targeting Stx is another method to detect the existence of non-O157 STEC. The advantage of this method is that it targets all strains of STEC and it is often used as "golden standard" to detect STEC infection. However, false positives can also be observed when other bacteria or free phage are present.

Due to the importance of O26, O45, O91, O103, O111, O113, O121, and O145 STEC, numerous methods targeting genes or antigens of lipopolysaccharide have been developed. These methods include PCR, real-time PCR, Luminex array, immunomagnetic separation (IMS), loop-mediated isothermal amplification and so on. Although these methods can rapidly detect specific *E. coli* serotypes. However, the *E. coli* serogroups detected by these methods are not necessarily STEC because those serogroups can be non pathogenic or other pathogenic *E. coli*.

Project overview

As emerging pathogens, non-O157 can cause both life-threatening diseases and huge foodborne outbreaks. Some non-O157 STEC are considered as virulent as O157 STEC. The public health concern of non-O157 is continuously increasing. Most of the STEC research focuses on O157; non-O157 STEC are not well characterized as foodborne pathogens and there is an urgent need to differentiate pathogenic and non-pathogenic STEC. The objective of this study was to explore non-O157 STEC of its presence in food, pathogenicity islands, and molecular evolution. Three specific objectives were as follows:

1) To identify the presence and characterization of non-O157 STEC from retail meats. Meat, especially ground meat, is easily contaminated by STEC during processing. Thus, meat can serve as transmission vehicles for STEC. Although a lot of studies have been done on O157 in meat products, there is a paucity of data about non-O157 STEC contamination in meat in the United States. Thus, the first objective of this study is to determine the prevalence of non-O157 STEC from retail meat and explore its potential as a human pathogen.

2) To determine distribution of pathogenicity islands and its associated virulence genes in non-O157 STEC. Although more than 470 non-O157 serotypes have been isolated from humans, not all non-O157 STEC are created equal. The scientific base for this is not well understood. Pathogenicity island (PAI), which is normally highly prevalent in high pathogenic strains, but almost absent in non-pathogenic strains of the same or closely related species, may contribute to the virulence difference and be used as a marker to distinguish non-O157 STEC, causing severe disease with nonpathogenic or low pathogenic strains. Thus, it is important to determine the distribution of PAIs in non-O157 STEC.

3) To determine the phylogenetic relationship of non-O157 STEC

O157:H7 is proposed to be evolved from EPEC O55:H7. However, the evolutional relationship of non-O157 STEC has not been well unveiled. Among all non-O157 STEC, serogroups O26, O111 and O103 caused most of the known outbreaks and severe diseases. Identifying its phylogenetic evolution can provide us with a model to study the evolution of non-O157 STEC and to help understand the pathogenesis evolution of non-O157 STEC. Determination of non-O157 phylogenetic relationship can also help to set bases for new markers.

In the following chapters, three studies are presented for each objective.

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CHAPTER II: NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN RETAIL GROUND BEEF AND PORK IN THEWASHINGTON D.C. AREA

Abstract

The prevalence and characteristics of non-O157 Shiga toxin-producing *Escherichia coli* **(**STEC) in retail ground meat from the Washington D.C. area were investigated in this study. STEC from 480 ground beef and pork samples were identified using PCR screening followed by colony hybridization. The STEC isolates were serogrouped and examined for the presence of virulence genes (s*tx*1, *stx*2, *eae* and *hly*A), and antimicrobial susceptibility. PFGE was used to identify the clonal relationships of STEC isolates, and PCR-RFLP was employed to determine *stx* subtypes. In addition, the cytotoxicity of STEC isolates was determined using a Vero cell assay. STEC were identified in 12 (5.2%) of 231 ground pork and 13 (5.2%) of 249 ground beef samples. Among 32 STEC isolates recovered from the 25 samples, 12 (37.5%) carried *stx2dact* and 7 (21.9%) carried *hly*A, but none carried *eae*. Nine isolates were identified as O91, and 17 (53.1%) isolates were resistant to two or more antimicrobials. Verotoxicity was detected in 26 (81.3%) of the STEC isolates. Thus, the retail ground meat was contaminated with a heterogeneous population of non-O157 STEC, some of which were potential human pathogens.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne pathogens worldwide (1). Most STEC infections cause self-limiting diarrhea, but some can progress to life-threatening diseases such as hemolytic uremic syndrome (HUS). To date, more than 470 STEC serotypes have been reported to be associated with human illness (2). *E. coli* O157: H7 is the predominant serotype associated with outbreaks and sporadic cases of STEC infections in the United States (3). However, a growing number of non-O157 serotypes have also been linked to human illnesses, including HUS (4). In the United States, non-O157 STEC cause an estimated 112,752 cases of illness each year, whereas *E. coli* O157:H7 causes 63,153 cases per year (5). Depending on geographic location, a variety of non-O157 serotypes were isolated from patients with STEC infections (6, 7). As only a limited number of laboratories test for non-O157 STEC, the public health risk associated with non-O157 STEC is likely underestimated.

Shiga toxin-producing *E. coli* are defined by their production of one or more Shiga toxins (Stx). The Stx family consists of two groups: Stx1 and Stx2. Stx1 is highly homogenous and consists of Stx1a, Stx1c and Stx1d (8, 9), whereas, Stx2 contains several variants including Stx2a, Stx2c, Stx2d, Stx2dact, Stx2e, Stx2f and Stx2g (10). Certain Shiga toxin subtypes are highly associated with clinical syndromes (11). STEC strains carrying certain *stx2* genes were frequently associated with severe diseases such as hemorrhagic colitis (HC) and HUS (12). Additionally, Intimin, an outer membrane protein encoded by *eae* that resides in the locus of enterocyte effacement (LEE), is highly associated with STEC infections (13).

However, STEC strains lacking *eae* have also been isolated from patients with severe disease (14). Additional adhesion factors, such as Saa (STEC autoagglutinating adhesion), Iha (IrgA homologues adhesion), and LPF (long polar fimbriae), may contribute to the adhesion process of *eae*-negative STEC (15).

Shiga toxin-producing *E. coli* are transmitted to humans mainly through consumption of contaminated food and water (16). Ground meat presents a greater risk than intact muscle because it can be contaminated with STEC during processing, and the pathogens present inside the ground product are more likely to survive during cooking (17). However, limited information is available about non-O157 STEC contamination in retail ground meat in the United States. The objectives of this study were to determine the prevalence of non-O157 STEC in ground meat in the Washington D.C. area, and to characterize STEC isolates to determine their virulence potential.

Materials and Methods

Sampling, culture enrichment and PCR assay. From March 2009 to March 2010, 480 samples (249 ground beef and 231ground pork) were collected weekly from three grocery chain stores in the Washington D.C. area, USA. The USDA-FSIS enrichment method was used in this study with modification (18). Briefly, a 25 g portion of each sample was placed in a plastic filter bag with 225 ml of modified tryptic soy broth (mTSB; 30 g TSB base, 1.5 g bile salts No. 3 and 1.5 g dipotassium phosphate per liter of distilled water) (Becton Dickinson, Sparks, MD). After homogenizing in a stomacher (Seward, Bohemia, NY), each sample was incubated for 15 to 22 h at 42 °C in a water bath with shaking at 100 rpm. One milliliter of the broth culture was taken for DNA extraction using the InstaGene DNA extraction kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. A PCR assay described by Lin et al was used to identify *stx*-positive samples (19). It amplified *stx1a, stx1c, stx1d, stx2a, stx2c, stx2d, stx2dactive, stx2e, stx2f* (19-22). The PCR was performed in a 25 µl reaction mixture containing 3µl of DNA template, 2.5 µl of 10 x PCR buffer, 2 µl of 25 mmol l^{-1} $MgCl_2$, 2 µl 1.25 mmol l^{-1} dNTP mix, 0.125 µl of 5 U μl^{-1} AmpliTaq Gold DNA polymerase mix (Applied Biosystems, Branchburg, NJ) and 0.2 µl of 50 pmol μl^{-1} of each primer. Thermal cycle condition was used as previously described (19). *E. coli* O157:H7 EDL933 and *E. coli* K-12 were used as positive and negative controls, respectively, in all PCR assays.

Colony hybridization. A colony hybridization procedure targeting *stx* genes was used to identify suspect STEC as previous described (6). A DNA probe targeting

both *stx1* and *stx2* was prepared by labeling *stx*-PCR amplicons from *E. coli* EDL 933 using the PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. In order to isolate STEC, up to six hybridization positive colonies were randomly picked and grown at 37 °C overnight on LB agar (Becton Dickinson, Sparks, MD). Isolates were determined as *E. coli* using the Vitek 2.0 system (bioMerieux, Marcyl'Etoile, France).

Molecular characterization of virulence genes. Two multiplex PCRs were used to determine the presence of *stx*1, *stx*2, *eae*A and *hly*A in STEC isolates (15). PCRs were performed in a 25 µl reaction system with 0.2 µl of 50 pmol μl^{-1} of each primer. *stx* subtypes were determined using PCR-RFLP as described previously (23) with the following control strains: EDL933 (*stx1a* and *stx2a*), E32511 (*stx2c*), EH250 (*stx2d*), S1191 (*stx2e*), B2F1 (*stx2dactive*) and N15018 (*stx1c*). *stx2dact* was confirmed by PCR according to our previously reported method (25).

Pulsed-field gel electrophoresis (PFGE). PFGE was performed following the updated protocol for non-O157 from PulseNet (24). *Salmonella* Braenderup H9812 was used as control for PFGE. PFGE gel pictures were analyzed with Bionumerics Software (Applied Maths, Austin, TX) using dice coefficients and unweighted pair group method with a 1.5% band position tolerance.

Vero cell cytotoxicity assay. Shiga toxin production of STEC isolates was evaluated using a Vero cell cytotoxicity assay as previously described (15, 25). Briefly, Vero cells were grown in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% fecal calf serum (Phenix Research Product, Candler, NC) under 5% CO_2 at 37°C. STEC isolates were inoculated in LB broth (Becton Dickinson, Sparks, MD) and incubated at 37°C overnight with shaking at 100 rpm. After adjusting the cell concentration to 10^9 CFU/ml with LB broth, 2 ml of the culture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.45μm–pore-size membrane filter (Fisher HealthCare, Houston, TX). The filtrate was serially diluted (1:5) and 100μl of each dilution was added to each well that was preseeded with Vera cells. After incubation under 5% CO_2 at 37°C for 48 h, 200 μl of 2% formalin in 0.067 M phosphate-buffered saline (pH 7.2) was added to fix Vero cells. After 1 h, the fixed cells were stained with 0.13% crystal violet in 5% ethanol for 30 min. The color density of each well was measured using a Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT) at 600 nm. EDL933 and *E. coli* k-12 were used as positive and negative controls, respectively. All assays were conducted in triplicate and repeated independently three times.

Molecular serogrouping. Serogroups including O8, O26, O28, O45, O91, O103, O111, O121, O145, and O157 were screened using PCR assays with primers based on the *wzy* gene (Table II-1). Each PCR was performed in a 25 μl reaction mixture containing 2 μl of DNA template, 2.5 μl of 10 x PCR buffer, 2 μl of 25 mmol l^{-1} $MgCl_2$, 2 μl of 1.25 mmol l^{-1} dNTP mix, 0.125 μl of 5 U μl^{-1} AmpliTaq Gold

DNA polymerase mix (Applied Biosystems) and 0.2 µl of 25 pmol μl^{-1} of each primer. An annealing temperature of 55 °C was used for all PCRs. The following strains were used as positive controls: EDL933 (O157:H7), UMD141 (O26:H11), UMD144 (O45:H2), TW7990 (O103:NM), UMD168 (O111:NM), SJ19 (O121:H19), UMD248 (O8:H4), P1334 (O91:H21), UMD327 (O28:NM), and SJ23 (O145:NM). *E.coli* K12 was used as negative control for all PCR assays.

Antimicrobial susceptibility. The antimicrobial susceptibilities of the STEC isolates were determined using a broth microdilition method (Sensititre system; Trek Diagnostic systems, Westlake, OH) and interpreted according to Clinical and Laboratory Standards Institute (26). The following antimicrobials were used: tetracycline, streptomycin, sulfisoxazole, nalidixic acid, kanamycin, amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole. Quality control organisms were *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212.

Data analysis. Chi square or Fisher's exact tests and T-test were used to analysis the data using SAS9.2 (SAS Institute, Cary, NC). A p-value of < 0.05 was considered statistically significant for comparisons.

Results

Prevalence of STEC in ground beef and pork. Among the 249 ground beef and 231 ground pork samples, 21 (8.5%) beef and 31 (13.4%) pork samples were positive for *stx* gene(s) by the PCR screening. Positive broth samples were further analyzed for STEC using colony hybridization. Overall, STEC were isolated from 13 (5.2%) of the beef and 12 (5.2%) of the pork samples. There was no significant difference in the prevalence of *stx* genes and STEC between pork and beef samples. Multiple STEC isolates were recovered from several samples resulting in a total of 32 isolates (16 from beef and 16 from pork).

Molecular characteristic of STEC isolates. PCR assays revealed that 10 (31.3%) of the 32 STEC isolates contained *stx1*, whereas 22 (68.7%) carried *stx2* but none harbored both *stx1* and *stx2* (Figure II-1). *stx1* was significantly more prevalent in STEC isolates from pork (50.0%) than in isolates from beef (12.5%) ($P< 0.05$); and *stx2* was significantly more prevalent in STEC isolates from beef (87.5%) than in isolates from pork (47.1%) ($P< 0.05$). Of 10 STEC isolates carrying *stx1*, 9 were positive for *stx1a* and 1 for *stx1d*. Among 22 *stx*2-carrying STEC isolates, 8 were positive for *stx2a*, 3 for *stx2a* and *stx2dact*, 9 for *stx2dact*, and 2 for *stx2e*. None of the STEC isolates contained the *eae* gene, but seven isolates (21.9%) harbored *hlyA*. There was no significant difference in the carriage of *hlyA* between STEC recovered from ground beef (31.3%) and STEC from ground pork (17.6%). Of the seven *hlyA*-positive isolates, six carried *stx2*, only one carried *stx1*. Nine isolates were identified

as serogroup O91, including eight from pork and one from beef (Figure II-1). The others did not belong to any of the other serogroups examined.

Pulsed-field gel electrophoresis (PFGE). A total of 26 distinct PFGE profiles were identified among the 32 STEC isolates, indicating heterogeneous STEC were present in the retail meat (Figure II-1). There was more than one PFGE pattern among STEC isolated from six samples (2, 158,197, 347, 348 and 380), suggesting that these samples were contaminated with multiple STEC clones. Additionally, identical PFGE profiles were found among STEC recovered from pork and beef samples (195-1, 196-6, 197-1 and 199-4; 126-1 and 419-1). Identical PFGE patterns were also obtained with different samples from the same source (347-2 and 348-1) (Figure II-1).

Vero cell cytotoxicity. Among the 32 isolates, 26 demonstrated toxicity to Vero cells, whereas 6 (3 from beef and 3 from pork) had no detectable cytotoxicity effect when compared with the negative control (Figure II-2). Three isolates (126-1, 245-1 and 460-10) displayed high cytotoxicity similar to that of the positive control strain *E. coli* O157:H7 EDL 933. All O91 isolates except 119-2 showed high cytotoxic effect to Vero cells. No significant difference in cytotoxicity between beef and pork STEC isolates was observed.

Antimicrobial susceptibility. Eighteen of the 32 STEC isolates (56.3%) were resistant to one or more antimicrobials. Seventeen (53.1%) isolates were resistant to

tetracycline, 14 (43.8%) to streptomycin, 13 (40.6%) to sulfisoxazole, 4 (12.5%) to nalidixic acid and 3 (9.4%) to kanamycin (Table II-2). Significantly more pork isolates (75.0%) than beef isolates (37.5%) were resistant to one or more antimicrobials ($P < 0.05$). Among the pork isolates, 12 (75.0%) were resistant to tetracycline, 8 (50.0%) to sulfisoxazole, 10 (62.5%) to streptomycin, 2 (12.5%) to nalidixic acid and 3 (18.8%) to kanamycin. Of the beef STEC isolates, 5 (31.2%) were resistant to tetracycline, 5 (31.2%) to sulfisoxazole, 3 (18.8%) to streptomycin, and 2 (12.5%) to nalidixic acid. Resistance to streptomycin and tetracycline was significantly more common in pork than in beef isolates ($P < 0.05$). All isolates were susceptible to amikacin, amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole.

Discussion

Isolation of non-O157 STEC from meat products containing a large number of other bacteria is still a challenge, since non-O157 STEC strains show great genetic and biochemical diversity, and there is no unique phenotypic marker that can differentiate them from other *E. coli*. There was no standard method to detect non-O157 STEC in meat product at the time the study was conducted. An enrichment protocol used originally for detecting O157:H7 in meat products by USDA-FSIS was adapted to detect STEC in ground beef and pork (18). As we used the same meat samples for testing other pathogens, 25 g instead of 325 g of sample was taken for the culture enrichment and isolation of STEC. In order to support the growth of all STEC, novobiocin was not added as studies showed that nonvobiocin inhibits the growth of some non-O157 STEC (27, 28). Instead, bile salt No. 3 was added to inhibit Gram-positive bacteria, and a higher temperature (42 °C) was used to control the growth of other *Enterobacteriaceae* (29). *S*haking at 100 rpm was also employed to enhance the growth of non-O157 STEC, especially those that might be injured during meat processing. However, a study showed that shaking could inhibit the detection of O157:H7 (30). The above variations may have contributed to a possible lower recovery rate of non-O157 STEC in our study.

Colony hybridization was employed to increase isolation rates of STEC as it allows the simultaneously screening of 100-200 colonies. Overall, 21 (8.5%) beef and 31 (13.4%) pork samples were positive for *stx* gene, and STEC isolates were recovered from 13 (5.2%) and 12 (5.2%) beef and pork samples, respectively. Failure to isolate STEC from *stx*-positive samples is a common problem. There are several

possible reasons like low numbers of STEC in the meat sample, high levels of background bacteria, the presence of *stx* carrying phages, the loss of *stx* during subculture of the bacteria, or the presence of other bacteria carrying *stx* (1, 31).

Contamination of STEC in ground beef has been studied by researchers around the world and it has been shown that the prevalence of non-O157 STEC ranges from 2.4% to 30% (32). A recent study of non-O157 STEC in the United States showed that 7.3% ground beef samples were positive for non-O157 STEC (33). Sekala et al. reported a prevalence of 5.5% STEC from 165 ground beef samples in Canada, in which 3.03% (5/165) were non-O157 STEC (34). In France, 3.9% of 411 beef samples were contaminated by non-O157 STEC (35). A much higher prevalence of non-O157 STEC was reported in ground beef samples in France (11%) (36) and Australia (16%) (17). The present study had a positive rate of 5.2% after testing 249 ground beef samples, and was in agreement with some of these reports. However, it is very difficult to compare different studies as geographical location, sampling, isolation and detection methods can affect the prevalence data significantly.

Compared to beef, fewer reports on the contamination of STEC in pork are available. In the present study, 12 (5.2%) pork samples were positive for non-O157 STEC, which was similar to the prevalence of non-O157 STEC in ground beef. We also identified 8 out of 16 STEC isolates from pork were O91, which has been associated with diarrhea and HUS (37). Read et al reported that 3.8% (9/235) ground pork samples were positive for non-O157 STEC in Canada (38), whereas only one STEC was isolated from 35 pork samples in New Zealand (39). In Austria, 1.7% of

120 pork samples were positive of STEC (40). More recently, 2.0% of 201 pork samples were found positive for STEC in Korea (41). These studies indicated that pork may also be a vehicle for transmission of STEC to humans.

Shiga toxin subtypes have been highly associated with clinical syndromes. Stx2a and Stx2dact are linked with high virulence and ability to cause severe disease. In this study, 9 out of 32 STEC isolates from retail meat harbored *stx2dact*, 3 with both *stx2a* and *stx2dact*. Although these STEC did not carry *eae*, they could still cause severe disease and outbreaks (42, 43). Only limited data on the presence of *stx2dact*-positive STEC in food are available. We previously reported that 5 of 16 STEC recovered from retail ground beef harbored *stx2dact* (15), and that 7 out of 153 STEC from food, cattle and human carried *stx2dact*, of which 2 were from food (25). In addition, Gobius et al found that 4 of 63 STEC isolates from ground beef and 1 of 103 from lamb meat contained *stx2dact* (44). These findings indicated that *stx2dact*-positive STEC are not uncommon in meat products.

While most STEC in the present study showed Vero cytotoxicity, six isolates were not cytotoxic to Vero cells. One possible explanation could be that the expression of *stx* was at very low level in those isolates. Previous studies found that some STEC strains produced low levels of Stx that could not be detected using tissue culture (45). It is also possible that although some STEC carry intact *stx*, it is not expressed (45). Lack of *stx* expression has been reported in *stx*2g STEC (23, 46, 47). Additionally, it is possible that those *stx* genes were not phage born or were from defective phages (45).

STEC particularly *E. coli* O157:H7 often carry other virulence genes in addition to *stx* (s), including *eae* and *hlyA*. Although *eae* is considered essential for causing attaching and effacing lesions in human intestinal epithelial cells, it may not be necessary for STEC pathogenicity. STEC lacking *eae* have been reported to cause illnesses including life threatening HUS and foodborne outbreaks (48, 49). None of the 32 STEC isolates in the present study carried *eae*, but nine belonged to O91, a STEC serogroup associated with clinical cases (37, 50). O91 is one of the most common serogroups isolated from adult patients (37). Additionally, the O91 STEC isolates except 119-2 demonstrated high cytotoxicity for Vero cells, indicating the potential as human pathogens.

While most *E. coli* O157:H7 are susceptible to antimicrobials, many non-O157 STEC isolated from humans and animals have shown resistance to multiple antimicrobials (51). More than half of the STEC isolates in the current study were resistant to two or more antimicrobials. Tetracycline, sulfisoxazole and streptomycin were the most common antimicrobials to which STEC were resistance to, similar to the findings of other studies (52-54). A greater resistance level was found in STEC from ground pork (12/16) than from ground beef (6/16). This is likely due to differences in animal husbandry practices in the raising of hogs and cattle for meat.

This study showed that ground beef and pork can be contaminated with heterogeneous STEC, many of which were resistant to commonly used antimicrobials. Some STEC belonged to serogroup associated with human illness and showed significant Vero cell cytotoxicity, suggesting their potential to cause illness in

humans. Education of consumers and food handlers on STEC in food are needed to reduce the risk of STEC infections in humans.

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TABLE II-1. Oligonucleotide primers based on wzy for PCR serogrouping.

Serogroup	Primer	Size	Accession Number
O8	5'-ACACCACAAACGATAATCAATGCC-3'	272	AF013583.1
	5'-GACCGATTAACAGCCAACACAGGT-3'		
O26	5'-TAATTGGCTTGCTGGGTTTATTCC-3'	422	AF529080.1
	5'-AGAATGAACCTTAATGCCATCAGC-3'		
O28	5'-GTCCAAAACGCAAGACGGTTC-3'	387	DQ462205.1
	5'-CCATACGCACGAGTGAATGTCC-3'		
O45	5'-GGCTCATCATTTGGTGCTTTGTG-3'	404	AY771223.1
	5'-ATAAGGATTTTCAGCGCCCCTG-3'		
O91	5'-CTGGAATGCTTGATGAACCTGGG-3'	28	AY035396.
	5'-AAGCCCCGACTCACTGTCAGAAAT-3'		
O103	5'-TTATACAAATGGCGTGGATTGGAG-3'	385	AY532664.1
	5'-TGCAGACACATGAAAAGTTGATGC-3'		
O111	5'-TAGGGGGCAGATTTTATATTCCGT-3'	379	AF078736.1
	5'-AACCAATGCTCCTATCACACCAAT-3'		
O121	5'-AGTGGGGAAGGGCGTTACTTATC-3'	366	AY208937.1
	5'-CAATGAGTGCAGGCAAAATGGAG-3'		
O145	5'-CCTGTCTGTTGCTTCAGCCCTTT-3'	392	AY863412.1
	5'-CTGTGCGCGAACCACTGCTAAT-3'		
O157	5'-TCGTTCTGAATTGGTGTTGCTCA-3'	278	AF061251.1
	5'-CTGGTGTCGGAAAGAAATCGTTC-3'		

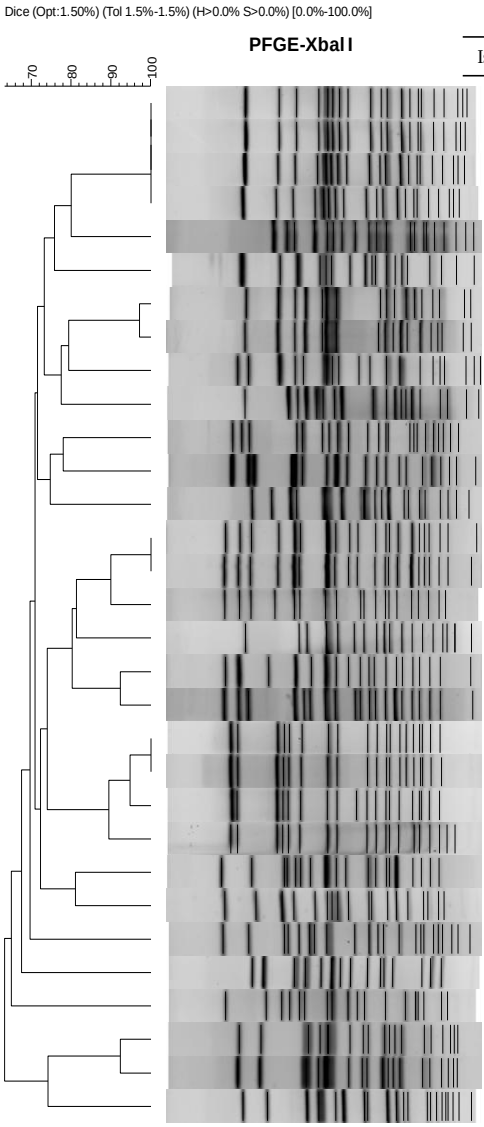
TABLE II-2. Antimicrobial resistance profiles of non-O157 STEC recovered from retail ground beef and pork.

Antimicrobial resistance pattern[a]	No. (%) Isolates from beef	No. (%) isolates from pork
NAL-STR-FIS-TET	1 (6.25%)	2 (12.50%)
KAN-STR-FIS-TET	0 (0.00%)	1 (6.25%)
NAL-FIS-TET	1 (6.25%)	0 (0.00%)
KAN-FIS-TET	0 (0.00%)	1 (6.25%)
KAN-STR-TET	0 (0.00%)	1 (6.25%)
STR-FIS-TET	1 (6.25%)	3 (18.75%)
STR-TET	1 (6.25%)	2 (12.50%)
FIS-TET	2 (12.50%)	1 (6.25%)
FIS	0 (0.00%)	1 (6.25%)
Total	6 (37.50%)	12 (75.00%)

[a]KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline.

FIGII-1. Dendrogram of PFGE profiles with XbaI of 32 STEC isolates from ground beef and pork.

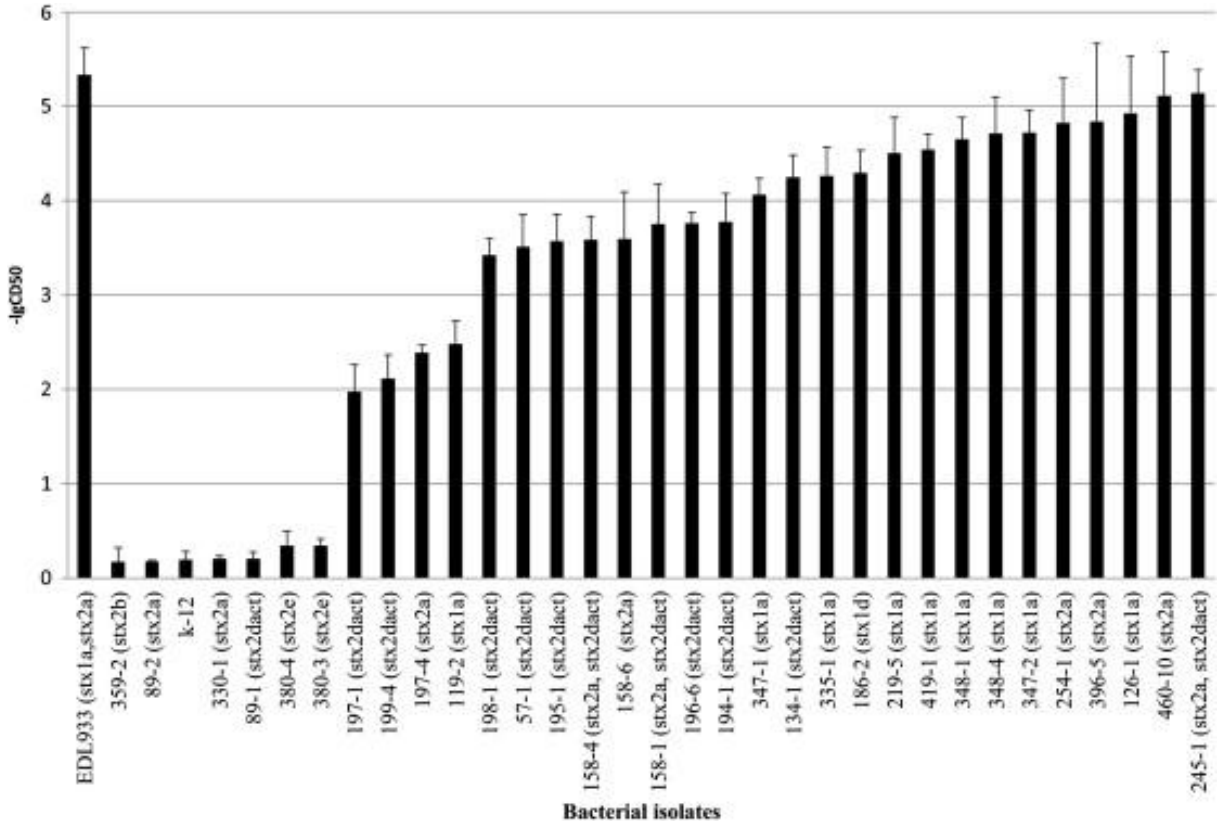
Similarity of the PFGE profiles was based on the Dice algorithm with 1.5% tolerance. (UN: unidentified by PCR for *E. coli* serogroups O8, O26, O28, O45, O91, O103, O111, O121, O145, and O157)



Isolates No.	Source	Serogroup*	*stx* subtype	*hlyA*
195-1	Ground beef	UN	*stx2dact*	-
196-6	Ground beef	UN	*stx2dact*	-
197-1	Ground pork	UN	*stx2dact*	-
199-4	Ground pork	UN	*stx2dact*	-
380-4	Ground pork	UN	*stx2e*	-
254-1	Ground beef	UN	*stx2a*	+
158-1	Ground beef	UN	*stx2a, stx2dact*	-
158-4	Ground beef	UN	*stx2a, stx2dact*	-
57-1	Ground beef	UN	*stx2dact*	-
330-1	Ground beef	UN	*stx2a*	-
134-1	Ground beef	UN	*stx2dact*	+
245-1	Ground beef	UN	*stx2a, stx2dact*	+
158-6	Ground beef	UN	*stx2a*	-
126-1	Ground beef	O91	*stx1a*	-
419-1	Ground pork	O91	*stx1a*	+
119-2	Ground pork	O91	*stx1a*	-
186-2	Ground beef	UN	*stx1d*	-
335-1	Ground pork	O91	*stx1a*	-
396-5	Ground pork	O91	*stx2a*	+
347-2	Ground pork	O91	*stx1a*	-
348-4	Ground pork	O91	*stx1a*	-
347-1	Ground pork	UN	*stx1a*	-
348-1	Ground pork	O91	*stx1a*	-
219-5	Ground pork	O91	*stx1a*	-
460-10	Ground beef	UN	*stx2a*	-
89-1	Ground beef	UN	*stx2dact*	-
359-2	Ground pork	UN	*stx2b*	-
89-2	Ground beef	UN	*stx2a*	-
194-1	Ground beef	UN	*stx2dact*	+
198-1	Ground pork	UN	*stx2dact*	+
197-4	Ground pork	UN	*stx2a*	-

FIG II-2. Vero cell cytotoxicity of 32 STEC isolates from ground beef and pork.

(CD50 = toxin dilution needed to cause 50% of cell detachment compared with untreated cell. CD50 value for each strain was an average of three independent assays. Values on the Y axis indicate the log of the reciprocal of CD50 values. *E. coli* O157:H7 EDL933 and *E. coli* K12 were used as positive and negative controls, respectively)


-lgCD50
0
1
2
3
4
5
6
EDL933 (stx1a,stx2a)
359-2 (stx2b)
89-2 (stx2a)
k-12
330-1 (stx2a)
89-1 (stx2dact)
380-4 (stx2e)
380-3 (stx2e)
197-1 (stx2dact)
199-4 (stx2dact)
197-4 (stx2a)
119-2 (stx1a)
198-1 (stx2dact)
57-1 (stx2dact)
195-1 (stx2dact)
158-4 (stx2a, stx2dact)
158-6 (stx2a)
158-1 (stx2a, stx2dact)
196-6 (stx2dact)
194-1 (stx2dact)
347-1 (stx1a)
134-1 (stx2dact)
335-1 (stx1a)
186-2 (stx1d)
219-5 (stx1a)
419-1 (stx1a)
348-1 (stx1a)
348-4 (stx1a)
347-2 (stx1a)
254-1 (stx2a)
396-5 (stx2a)
126-1 (stx1a)
460-10 (stx2a)
245-1 (stx2a, stx2dact)
Bacterial isolates

CHAPTER III: DISTRIBUTION OF PATHOGENICITY ISLANDS OI-122, OI-43/48, OI-57 AND HIGH PATHOGENICITY ISLAND (HPI) IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

Abstract

Pathogenicity islands (PAIs) play an important role in STEC pathogenicity. The distribution of PAIs OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) were determined among 98 STEC strains assigned to seropathotypes (SPT) A to E. PCR and PCR-RFLP assays were used to identify 14 virulence genes that belonged to the four PAIs, and to subtype *eae* and *stx* genes, respectively. Phylogenetic trees were constructed based on the sequences of *pagC* among 34 STEC strains and *iha* among 67 diverse pathogenic *E. coli*, respectively. Statistical analysis demonstrated that the prevalence of OI-122 (55.82%) and OI-57 (82.35%) was significantly greater in seropathotypes (SPT A, B and C) that are frequently associated with severe disease than in other seropathotypes. *terC* (62.5%) and *ureC* (62.5%) in OI-43/48 were also significantly more prevalent in SPT A, B and C than in SPT D and E. Additionally, OI-122, OI-57, OI-43/48 and their associated virulence genes (except *iha*) were found to be primarily associated with *eae*-positive STEC, whereas HPI occurred independently of the *eae* presence. The strong association of OI-122, OI-43/48 and OI-57 with *eae*-positive STEC suggests in part different pathogenic mechanisms exist between *eae*-positive and *eae*-negative STEC. Virulence genes in PAIs that are associated with severe diseases can be used as potential markers to aid in identifying highly virulent STEC.

Introduction

Shiga toxin-producing *Escherichia coli* can cause human illnesses ranging from self-limiting diarrhea to life-threatening diseases such as hemolytic uremic syndrome (HUS), a leading cause of kidney failure in children (1). *E. coli* O157:H7 is the single serotype that causes most STEC outbreaks and HUS cases. Like O157, non-O157 STEC can also cause severe diseases and foodborne outbreaks (1). There are more than 470 non-O157 STEC serotypes have been associated with human illness (2) and the public health concerns of non-O157 STEC is increasing (1). Estimations indicate that non-O157 STEC cause 112,752 illness each year in the United States, almost doubling the numbers of O157:H7 illnesses (63,153) (3). While some non-O157 STEC have been associated with disease symptoms indistinguishable from O157:H7, not all STEC can cause HUS and outbreaks, and some STEC serotypes have never been reported related to any human illness (4). The scientific basis for this difference, however, is poorly understood.

Increasing evidence shows that differences in virulence between pathogenic and nonpathogenic bacterial strains can be attributed in part to virulence genes located in pathogenicity islands (PAIs) (5). PAIs usually contain blocks of virulence genes and are greater than 10 Kb (6). Several PAIs have been identified and characterized in STEC. A chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) was identified in *E. coli* O157:H7 strain EDL933, which encodes a type III secretion system (TTSS) as well as virulence genes (*eae* and *tir*) associated with the intimate attachment of bacteria to intestinal epithelial cells (4). LEE appears to confer enhanced virulence, since LEE-positive STEC are much more

commonly associated with HUS and outbreaks than LEE-negative STEC (5). However, some LEE-positive STEC serotypes have never been associated with disease, and some LEE-negative STEC can cause HUS and outbreaks, indicating that virulence factors other than those in LEE may contribute to pathogenesis of STEC (5).

Pathogenicity island OI-122 is also well characterized in O157:H7 (5, 7). OI-122 is a 23 kb PAI consisting of three modules (5, 8, 9). Z4321 is located in module one and encodes a protein sharing 46% similarity with the *phoP*-activated gene C (*pagC*) of *Salmonella* Typhimurium (5, 9). Z4326, Z4328, and Z4329 are located in module two. Z4326 (*sen*) encodes a protein that shares 38.2% similarity to *Shigella flexneri* enterotoxin 2 (5), whereas Z4328 and Z4329 encode proteins that have 89% and 86% similarity to non-LEE encoded effectors NleB and NleE, respectively (9). The enterohemorrhagic *E. coli* factor for adherence (Efa), which is involved in epithelial cell adhesion and proliferating the inhibition of bovine peripheral blood lymphocytes, is located in module three (10).

OI-43 and OI-48 are duplicate genomic islands found in EDL933 (8). OI-43/48 genes are divided into three functional groups: a seven-gene cluster *ureDABCEFG* that encodes urease and accessory proteins hydrolyzing urea to ammonia and carbon dioxide; telluride resistance genes *ter*ZABCDEF (11); and two putative adhesion genes, *iha* (iron-regulated gene A) and *aidA-1* (autotransporter adhesin involved in diffuse adherence) (12).

In EDL933, OI-57 contains non-LEE encoded effector genes *nleG2-3*, *nleG6-2*, and *nleG 5-2* (13, 14). NleG proteins are E3 ubiquitin ligases analogous to RING finger and U-box enzymes in eukaryotes. Although the exact functions of NleG2-3, NleG6-2, and NleG 5-2 are still unclear, similar proteins have been identified as effectors that suppress immune response from the host (15).

High pathogenicity island (HPI) was first detected in *Yersinia pestis* and other highly virulent *Yersinia* species, and encodes a siderophore (yersiniabactin) mediated iron-uptake system (16). HPI is required for full virulence expression in *Yersinia* (16), and contains two main virulence genes, *fyuA* and *irp2*. FyuA is an outer membrane protein acting as a receptor for ferric-yersiniabactin uptake and for bacteriocin pesticin, while Irp2 is involved in yersiniabactin synthesis (17). An orthologous and highly conserved HPI is widely distributed among different species and genera of the family *Enterobacteriaceae* (16).

Few studies have investigated PAIs other than LEE in STEC to date. Since PAIs are normally absent in non-pathogenic strains of the same or closely related species, they may serve as useful markers to distinguish highly virulent from less virulent or harmless strains (5, 6) . In addition, PAIs can be used to identify new and emerging pathogenic bacteria. In this study, we reported the distribution of OI-122, OI-43/48, OI-57, and HPI and their virulence genes in STEC, and evaluated the association of the PAIs and individual virulence genes with STEC seropathotypes linked to severe diseases and outbreaks. In addition, the association of the four PAIs with LEE was determined.

Materials and Methods

Bacterial strains. A total of 98 STEC strains from humans, animals and food were used in this study (TABLE III-1). Strains were classified into seropathotypes A to E according to the criteria described by Karmali *et al.* (5). The assignment of seropathotypes was based on published references (5, 14, 18) and a large online database on non-O157 STEC (http://www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm).

***stx* and *eae* subtyping**. *stx* and *eae* subtypes were determined using PCR-RFLP analysis (19, 20), and *stx2dact* was confirmed by PCR as previously described (21). Genomic DNA was extracted using boiling method as previously described (22, 23). STEC strains S1191 (*stx2e*), EDL933 (*stx1a* and *stx2a*), E32511 (*stx2c*), EH250 (*stx2b*), B2F1 (*stx2dact*) and N15018 (*stx1c*) were used as positive controls for the *stx* subtyping; STEC strains 86-24 (gamma 1), EDL933 (gamma 1), TW06584 (kappa), E2348-69 (alpha), TW07920 (epsilon), RDEC-1 (beta), TW10366 (rho), TW03501 (iota), TW07892 (eta), and TW01387 (gamma 2/theta) were used as positive controls for the *eae* subtyping. *E. coli* K12 was used as a negative control strain for both *stx* and *eae* subtyping.

Presence of OI-122, OI-43/48, O-57 and HPI. PCR assays were used to determine the presence of 14 virulence genes in STEC OI-122, OI-43/48, O-57 and HPI as described (5, 11, 13, 24, 25). The presence of a PAI was determined by several marker genes located in different regions of the island, including *pagC, sen, nleB, efa-1,* and *efa-2* for OI-122; *terC, ureC, iha* and *aidA-1* for OI-43/48; *nle2-3, nleG6-2* and *nleG5-2* for OI-57; and *irp2* and *fyuA* for HPI. PCR was performed in a 25 µl reaction mixture, containing 2 µl of DNA template, 2.5 µl of 10x PCR buffer, 2

µl of 25 mmol l^{-1} $MgCl_2$, 2 µl of 1.25 mmol l^{-1} dNTP mix, 0.125 µl of 5 U $µl^{-1}$ AmpliTaq Gold DNA polymerase mix (Applied Biosystems, Branchburg, NJ) and 0.2 µl of 50 pmol $µl^{-1}$ of each primer. *E. coli* O157:H7 EDL 933 was used as a positive control for the virulence genes of OI-122, OI-43/48 and OI-57, and *E. coli* O26:H11 SJ-13 for virulence genes of HPI. *E. coli* K12 was used as a negative control for all PCR assays.

Phylogenetic and sequences analysis. *iha* and *pagC* were the only two genes that were highly prevalent in both *eae*-positive and *eae*-negative STEC. To determine the evolutionary relationship between the two groups of STEC, *iha* and *pagC* were selected for phylogenetic analysis studies. *iha* sequences from 67 *E. coli* and *Shigella* were obtained from the GenBank. A multiple sequence alignment of *iha* was performed using ClustalW in MEGA 5.05, and a maximum likelihood phylogenetic tree was generated using the General Time Reversible model (26). A bootstrapping of 2,000 replicates was used to estimate the confidence of the branching patterns of the phylogenetic tree using *iha* of *E. coli* SMS-3-5 as the phylogenetic tree's root.

Additionally, PCR was used to amplify *pagC* of OI-122 from 12 selected STEC strains representing different serotypes as described by Konczy *et al* (9). PCR products were sequenced by GeneWiz (Germantown, MD). Twenty two *pagC* sequences representing different STEC serotypes and one *Citrobacter* were downloaded from the GenBank. The *pagC* sequences were cropped to 446 bp prior alignment. Phylogenetic analysis was performed using ClustalW within MEGA 5.05 (26). A phylogenetic tree based on *pagC* sequences was constructed using maximum

likelihood methods by MEGA 5.05 with bootstrapping of 2,000 replicates using *pagC* of *Citrobacter rodentium* IC168 as the tree's root.

Statistical analysis. Chi square or Fisher's exact test was used for data analysis using SAS9.2 (SAS Institute, Cary, N.C.). A P-value of < 0.01 was considered statistically significant.

Results

Distribution of OI-122, OI-43/48, OI-57 and HPI in STEC. The 98 STEC strains were classified into seropathotypes A to E (TABLE III-1). Overall, the prevalence of OI-122 and OI-57 decreased progressively from seropathotype A (SPT A) to seropathotype E (SPT E) (FIG III-1). The prevalence of OI-122 and OI-57 was significantly higher in seropathotypes associated with severe diseases (SPT A, B, and C) and outbreaks (SPT A and B) than in other seropathotypes ($P<0.0001$) (TABLE III-2). Although the prevalence of OI-43/48 was greater in seropathotypes associated with HUS (SPT A, B and C) and outbreaks (SPT A and B) than in other seropathotypes, the difference was not statistically significant ($P= 0.1356$ and 0.02, respectively). HPI was not found in SPT A (O157), but was almost evenly distributed from SPT B to SPT E (FIG III-1).

sen, nleB, efa-1, efa-2, terC, ureC, nleG2-1, nleG5-2 and *nleG6-2* were significantly more prevalent in SPT A and B than in SPT C, D and E (TABLE III-3). *pagC, sen, nleB, efa-1, efa-2, terC, ureC, nleG2-1, nleG5-2* and *nleG6-2* were statistically more prevalent in SPT A, B and C than in SPT D and E (TABLE III-3). Although *aidA-1* was more prevalent in SPT A and B than in SPT C, D and E, the difference was not statistically significant ($P=0.27$). *iha, fyuA,* and *irp2* were less prevalent in SPT A, B and C than in SPT D and E, and the differences were not statistically significant neither.

Distribution of OI-122, OI-43/48, OI-57 and HPI in EHEC O157. PAIs showed three patterns of distribution in EHEC O157 (TABLE III-4). As to β-

glucuronidase (GUD)-negative O157:H7, four strains all contained marker genes for OI-122, OI-57, and OI-43/48. In GUD-positive O157: H7, none of the five strains carried *efa-1* and *efa-2* (located at the third module of OI-122) or *aidA-1*(located at the end of OI-43/48). Sorbitol fermenting O157:NM strains contained all virulence genes of OI-122 and OI-57 but were negative for all the OI-43/48 virulence marker genes, indicating the absence of OI-43/48 in O157:NM. Additionally, none of the O157:H7 and O157:NM strains were positive of HPI virulence genes.

Association of OI-122, OI-43/48, OI-57 and HPI with *eae*. We compared the distribution of virulence genes of OI-122, OI-43/48, OI-57, and HPI between *eae*-positive and *eae*-negative STEC. All virulence genes of OI-122 and OI-57 (*pagC, sen, nleB, efa-1, efa-2, nleG2-3, nleG5-2* and *nleG6-2*) were highly prevalent in *eae*-positive strains (FIG III-2). However, these genes, with the exception of *pagC*, were less prevalent in *eae*-negative STEC (FIG III-2). Although 38.6% of *eae*-negative STEC strains were positive for *pagC*, its prevalence was significantly higher (64.8%) in *eae*-positive STEC (P=0.005). There was no apparent physical or functional relationship identified between OI-43/48 and LEE, but three OI-43/48 virulence genes (*ureC, terC* and *aidA-1*) were mainly associated with the presence of *eae* (P<0.0001). On the other hand, *iha* was more prevalent in *eae*-negative than in *eae*-positive STEC strains (P=0.007). As for HPI, there were no significant differences in the distribution of *fyuA* or *irp2* between *eae*-positive and *eae*-negative STEC (P=0.36).

Phylogenetic analysis of *iha* from diverse pathogenic *E. coli*. A phylogenetic tree based on *iha* separated *eae*-positive and *eae*-negative STEC strains into two distinct clades (FIG III-3). In clade I, two subgroups, Ia and Ib, shared at least 98.0% sequence similarity. *eae*-positive EHEC serotypes highly associated with outbreaks and severe diseases were located in clade I (O157:H7, O26:H11, O103:H2, O111:NM, O145:H28). Those sequences shared at least 99% similarity and clustered together with *iha* from other pathogenic *E. coli* including enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), eneteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and Shiga toxin-producing EAEC O104:H4 (from a German outbreak in 2011). *iha* sequences from the O26:H11, O111:H11, and O111:NM strains formed subgroup Ib and shared at least 98.0% sequence similarity with subgroup Ia. Interestingly, strains DEC10A (O26:H11), DEC10C (O26:H11), 11368 (O26:H11) and DEC8C (O111:NM) carried two *iha* that clustered separately in Ia and Ib.

All 15 *iha* sequences from *eae*-negative STEC clustered together to form clade II. Multiple sequence alignments demonstrated that *iha* from *eae*-negative STEC shared only 91.1-93.6% sequence similarity with *iha* from clade I. *iha* from subgroups IIa and IIb shared only 93.8-94.3% sequence similarity. As in some *eae*-positive strains, *eae*-negative STEC strains CL-3 (O113:H21), 96.0497 (O91:H21) and B2F1 (O91:H21) also carried two *iha* genes that clustered separately in subgroups IIa and IIb.

Phylogenetic and sequence analysis of *pagC*. The *pagC* phylogenetic tree showed four clades (FIG III-4). The *eae*-positive *E. coli* STEC formed a single clade with EPEC and ETEC, whereas the *eae*-negative STEC strains formed two clades, along with one strain that clustered with a *Citrobacter rodentium* strain. We identified 15 single nucleotide polymorphisms (SNPs) and one indel among the 35 *pagC* sequences. Sequence analysis revealed that an insertion of adenine at nucleotide 388 in two O103:H25, two O45:H2, one O103:H2 and one O103:H6 strains led to a frame shift mutation, and that a premature stop codon truncated the protein at the third loop resulting in the loss of the fourth and last loops.

Discussion

In the present study, PAIs OI-122 and OI-57 of STEC were found highly associated with seropathotypes that can cause severe disease and outbreaks, as previously demonstrated (7, 13, 14). Several OI-122 virulence factors play important roles in bacterial pathogenesis. For example, PagC can promote the survival of *Salmonella* within macrophages (5, 9). Efa is an adhesion protein originally described in some EHEC strains (27). The *efa-1* gene is almost identical to *lifA*, an EPEC gene encoding lymphostatin (LifA) (28), which inhibits the proliferation of mitogen-activated lymphocytes and the synthesis of proinflammatory cytokines (28). Efa1/LifA also contributes to EPEC adherence to epithelial cells and is critical for the intestinal colonization by *C. rodentium* (29). NleB was required for full colonization and colonic hyperplasia in mice and a mutation of *nleB* abolished lethality of *C. rodentium* in C3H/HeJ mice (7, 30).

Whereas OI-122 is highly related to colonization and suppression of the host immune system, the function of OI-57 is largely unknown. Wu *et al.* (15) determined that NleG like proteins including NleG2-3, NleG6-2 and NleG5-1 were E3 ubiquitin ligases, analogous to RING finger and U-box enzymes in eukaryotes. Although the targets of the OI-57 Nle effectors are unknown, several similar effectors are primarily involved in suppressing host immune response by degrading immune-related host proteins (15). Thus, it is possible that OI-57, similar to OI-122, would be also related to suppression of host immune system.

In addition to the virulence genes in OI-122 and OI-57, *ureC* and *terC* located on OI-43/48 were also highly associated with seropathotypes related to severe disease

and outbreaks. Urease has been confirmed as an important virulence factor in several bacterial species, such as *Helicobacter pylori*, *Yersinia enterocolitica*, *Proteus mirabilis*, *Brucella* species, and *Klebsiella pneumonia* (31). Mutation of *ureC* has led to a reduced adherence of EHEC O157:H7 in ligated pig intestine (12). A recent study by Steyert *et al* (32) revealed that strains with non-functional urease were two times unlikely to survive passage through the stomach and had a reduced ability to colonize the mouse intestinal tract compared with urease-positive strains. These data demonstrate that urease can help STEC strains survive in the stomach and enhance its competitiveness in colonization in calf and human intestinal tracts. The role of tellurite resistance genes (*ter*ZABCDEF) in STEC is still not well understood. Yin et al (12) showed that mutation of the *ter* cluster in O157:H7 led to fewer adherence to epithelial cells and smaller bacterial clusters compared with wild-type strains. Therefore, *ter* genes might encode an adhesin or a gene product that promotes the function of adhesion(s). In addition, tellurite salts are strong oxidative agents, and it is possible that *ter* genes might offer a selective advantage in the host environment and aid STEC in general stress response (12).

Interestingly, *ureC* has been more frequently found in *eae*-positive STEC (113/132) than in *eae*-negative strains (4/70) although no physical linkage of *ure*C and *eae* has been identified (33). The prevalence of *ureC* in *eae*-positive STEC (45/55) was significantly higher than in *eae*-negative STEC (2/44) ($P < 0.0001$). Similarly, *terC* was also more prevalent in *eae*-positive STEC (45/55) than *eae*-negative strains (5/55) ($P < 0.0001$). Even though OI-43/48 and LEE are physically

distant, our observations indicated that there might be a functional relationship between LEE and OI-43/48.

The arrangement of OI-122 genes was found to be serotype dependent and all O157:H7 strains have a complete OI-122 (5, 9). However, we found that two patterns of OI-122 existed in O157:H7. An incomplete OI-122 lack of third module was identified in all GUD-positive O157:H7 strains. Additionally, *aidA-1* of OI-43/48 was absent in GUD-positive O157:H7.

Most OI-122, OI-43/48 and OI-57 virulence genes (*pagC*, s*en, nleB, efa-1, efa-2, terC, ureC, iha, aidA-1, nleG2-3, nleG6-2,* and *nleG5-2*) were highly prevalent in *eae*-positive STEC. However, they were largely absent in *eae*-negative STEC with the exception of *pagC* and *iha*. Phylogenetic analysis revealed that *iha* genes from *eae*-positive STEC had high similarity (99.6%), whereas they had lower sequence similarity (91.1-93.6%) with *iha genes* from *eae*-negative STEC, indicating that *iha* from *eae*-positive and *eae*-negative STEC may have evolved independently or have different origins. Such a difference also existed in *pagC* between *eae*-positive and *eae*-negative STEC. Schemidt *et al* (34) reported that *iha* was carried by a 33,014 bp PAI in STEC serotype O91:H- strains (*eae*-negative). In addition, *iha* was found in pO113 plasmid of STEC serotype O113:H21 (*eae*-negative) (35). Moreover, Shen *et al* (36, 37) reported that *pagC* was identified within a mosaic PAI from STECO113:H21strain CL-3 (*eae*-negative). Thus, the higher prevalence of *iha* and *pagC* in the *eae*-negative STEC strains, as compared with other virulence marker genes in this study, is likely due to the presence of the same or similar PAIs and/ or plasmids as previously described. The similar prevalence rate of *iha* genes in the

seropathotypes highly associated with severe diseases and other seropathotypes indicates that *iha* is not related with severe clinical outcomes, but the significantly higher prevalence of *pagC* in the seropathotypes associated with severe diseases indicates that this gene has some association with severe clinical outcome whether a strain carries the gene in OI-122 or in some other PAIs.

The distribution of PAI virulence genes and the phylogenetic analysis of *iha* and *pagC* support the hypothesis that OI-122, OI-43/48 and OI-57 are primarily associated with *eae*-positive strains in STEC. However, some *eae*-negative STEC serotypes, for example, O113:H21 and O91:H21, are also associated with life threatening diseases such as HUS (5). Virulence factors such as subtilase cytotoxin AB5 (subAB5) and Saa (STEC autoagglutinating adhesion) are more commonly associated with *eae*-negative STEC. Moreover, it has been shown that some LEE-negative STEC, especially O113:H21, can invade tissue culture cells (38).Whole genome comparison between nine *eae*-negative and five *eae*-positive STEC strains revealed that *eae*-negative strains did not carry any LEE or other phage encoded non-LEE effectors (39). These observations indicate that some differences in pathogenesis mechanisms may exist between *eae*-positive and *eae*-negative STEC. Additional studies, especially genomics and proteomics, are needed to unveil the difference in the pathogenicity mechanisms between *eae*-negative and *eae*-positive STEC.

The strong association of OI-122, OI-57 and OI-43 with *eae*-positive STEC offers an important basis for STEC molecular risk assessment (MRA). The MRA, which uses 14 non-LEE encoded virulence factors to distinguish high risk from low

risk non-O157 STEC, was proposed by Coombes *et al* in 2008 (13). Other researchers adopted this concept and applied it to their own studies (40-43). However, the current work demonstrated that some of non-LEE encoded effectors (*nleB, nle2-3, nleG5-2* and *nleG6-2*) were primarily associated with *eae*-positive STEC strains. In addition, Mundy et al (44) reported that *nleA* was present in 37 out of 43 (86%) *eae*-positive STEC, but absent in 50 *eae*-negative STEC clinical strains. Konczy *et al* (9) reported that *nleB* and *nleE* of OI-122 were highly correlated with LEE. Moreover, comparative genomics analysis demonstrated that all known phage encoded non-LEE effector genes were absent in *eae*-negative STEC (39). Based on the MRA framework, which uses non-LEE effector genes as sole markers, all *eae*-negative virulence STEC, including HUS associated O113:H21, O91:H21 and O104:H21, would be grouped as harmless STEC; other serotypes, for example, O103:H11 and O119:H25, which have not been reported as associated with severe disease or outbreaks but carry similar non-LEE encoded virulence effectors as O157 EHEC, would be considered as outbreak- and severe disease-associated serotypes. Therefore, additional markers or methods of assessment, especially for *eae*-negative STEC, are needed to accurately distinguish highly pathogenic STEC from low virulent or harmless STEC.

In summary, O-122 and OI-57, and their virulence genes were highly associated with seropathotypes that cause severe diseases and outbreaks. In addition, *ureC* and *terC* located at OI-43/48 were also identified as markers related to high risk seropathotypes. Virulence genes in PAIs that are associated with severe diseases can be used as markers to identify potentially highly virulent STEC. Furthermore, this

study demonstrated that OI-122, OI-43/48, and OI-57 are highly associated with *eae*-positive STEC, which offers an important basis for STEC MRA.

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TABLE II1-1. Distribution of *eae* subtypes, and virulence genes of OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) by serotypes

Seropathotype	Serotype	*eae* subtype	No. of strains	No. of strains positive by PCR for													
				pagC	*sen*	*nleB*	*efa-1*	*efa-2*	*terC*	*ureC*	*iha*	*aidA-1*	*nleG2-3*	*nleG5-2*	*nleG6-2*	*fyuA*	*irp2*
A	O157:H7 GUD-*	Gamma 1	4	5	5	5	5	5	5	5	5	5	5	5	5	0	0
	O157:H7 GUD+	Gamma 1	5	5	5	5	0	0	5	5	5	0	5	5	5	0	0
	O157:NM	Gamma 1	6	6	6	6	6	6	0	0	0	0	6	6	6	0	0
B	O26:H11	Beta	4	0	4	4	4	4	4	4	4	3	4	4	4	4	4
	O103:H2	Epsilon1	4	2	4	4	4	4	2	2	1	2	4	1	2	0	0
	O111:H8/NM	Gamma2/theta	4	3	4	4	4	4	4	4	4	0	4	3	4	2	2
	O121:H19	Epsilon1	3	3	3	3	3	3	3	3	0	0	3	3	3	0	0
	O145:H28/NM	Gamma1	4	1	4	4	3	3	4	4	4	4	3	2	3	0	0
C	O5:NM	Beta	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0
	O22:H5	negative	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1
	O22:H8	negative	2	1	0	0	0	0	0	0	2	0	0	0	0	0	0
	O45:H2	Epsilon1	4	4	4	4	4	4	4	4	0	4	4	3	3	0	0
	O50:H7	Epsilon1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0
	O55:H7	Gamma 1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
	O91:H21	Negative	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	O104:H21	Negative	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	O113:H21	Negative	4	4	0	0	0	0	0	0	4	1	0	0	0	0	0
	O118:H16	Beta	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
	O125:NM	Beta	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0
	O128:H2	Beta	1	0	0	0	0	0	0	0	1	1	0	0	0	1	1
	O128:H45	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	ONT:H2	Negative	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	OUN:NM	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
D	O2:H27	Negative	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0

	O15:H27	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1
	O91:H14	Negative	4	4	0	0	0	0	0	0	4	0	0	0	0	0	0
	O91:H7	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O103:H11	Beta	2	2	2	2	2	2	1	1	2	2	2	2	2	2	2
	O103:H25	Gamma2/theta	3	3	3	3	3	3	3	3	0	3	2	0	2	0	0
	O111:H11	Beta	4	0	4	4	4	4	4	4	4	3	4	4	4	4	4
	O116:H21	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O126:H8	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O146:H21	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O174:H8	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
E	O8:H16	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O8:H28	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O15:H16	Negative	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1
	O36:H14	Negative	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	O46:H38	Negative	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0
	O73:H18	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O83:H8	Negative	7	3	0	0	0	0	2	0	3	0	0	0	0	0	0
	O88:H38	Negative	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
	O88:H49	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	O113:H36	Negative	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	O128:H16	Negative	4	2	2	2	4	2	2	2	4	4	4	2	3	4	4
	O168:H8	Negative	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	O174:H36	Negative	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0

TABLE III-2. Distribution of pathogenicity islands (PAIs) in seropathotypes associated outbreak (SPT A and B) and severe disease (SPT A, B and C).

PAIs	Association of PAIs with seropathotypes related to outbreak		Association of PAIs with seropathotypes related to severe disease	
	Prevalence (%) in SPT A and B (n=34)	Prevalence (%) in SPT C, D and E (n=64)	Prevalence (%) in SPT A, B and C (n=56)	Prevalence (%) in SPT D and E (n=42)
OI-122	55.82*	17.18*	46.43 +	9.52 +
OI-43/48	32.35	12.50	26.79	14.28
OI-57	82.35*	21.86*	60.71 +	19.05 +
HPI	17.65	25.00	17.86	28.57

*: Statistically significant difference between SPT A and B with SPT C, D and E. +: Statistically significant difference between SPT A, B and C with SPT D and E. A P value less than 0.01 is considered as statistically significant.

TABLE III-3. Distribution of virulence genes located at pathogenicity islands (PAIs) in seropathotypes associated outbreak (SPT A and B) and severe disease (SPT A, B and C).

Gene	PAIs	Association of virulence genes with seropathotyps related to outbreak		Association of virulence genes with seropathotyps related to severe disease	
		Prevalence (%) in SPT A and B (n= 34)	Prevalence (%) in SPT C, D and E (n=64)	Prevalence (%) in STEC SPT A, B and C (n=56)	Prevalence (%) in STEC in SPT D and E (n=42)
pagC	OI-122	70.59	46.88	69.64 +	35.71 +
sen	OI-122	100.00 *	31.25 *	76.79 +	26.45 +
efa-1	OI-122	82.35 *	31.25 *	66.07 +	30.95 +
efa-2	OI-122	82.35 *	31.25 *	66.07 +	26.45 +
nleB	OI-122	100.00 *	31.25 *	76.79 +	26.45 +
terC	OI-43/48	76.47 *	35.94 *	62.50 +	33.33 +
ureC	OI-43/48	76.47 *	31.25 *	62.50 +	26.45 +
Iha	OI-43/48	64.70	70.31	64.29	73.80
aidA-1	OI-43/48	38.23	32.81	39.28	28.57
nleG2-3	OI-57	97.06 *	31.25 *	73.21 +	28.57 +
nleG5-2	OI-57	82.35 *	21.88 *	60.71 +	19.04 +
nleG6-2	OI-57	97.18 *	28.13 *	66.07 +	28.57 +
fyuA	HPI	17.65	23.44	16.07	28.57
irp2	HPI	17.65	23.44	16.07	28.57

*: Statistically significant between SPT A and B with SPT C, D and E. +: Statistically significant between SPT A, B and C with SPT D and E. A P value less than 0.01 is considered as statistically significant.

TABLE III-4. Characterization and presence of PAIs associated virulence genes among O157:H7 and O157:NM

				OI-122					OI-43/48				OI-57			HPI	
Strains	Serotype	*stx*	GUD*	*pagC*	*sen*	*efa-1*	*efa-2*	*nleB*	*terC*	*ureC*	*iha*	*aid-1*	*nleG2-3*	*nleG6-2*	*nleG5-2*	*irp2*	*fyuA*
EC4115	O157:H7	*stx1,stx2*	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Saika	O157:H7	*stx1, stx2*	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
TW14359	O157:H7	*stx1, stx2*	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
FDA413	O157:H7	*stx2*	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
1628	O157:H7	*stx1,stx2*	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
1659	O157:H7	*stx1,stx2*	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
EC97144	O157:H7	*stx1,stx2*	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
EC96038	O157:H7	*stx1,stx2*	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
EC96012	O157:H7	*stx1,stx2*	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-
493/89	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
5412/89	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
IH56929	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
IH56909	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
H1085C	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-
H2687	O157:NM	*stx2*	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-

GUD: β-glucuronidase

FIG III-1. Distribution of OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) in STEC strains (n=98) of different seropathotype.

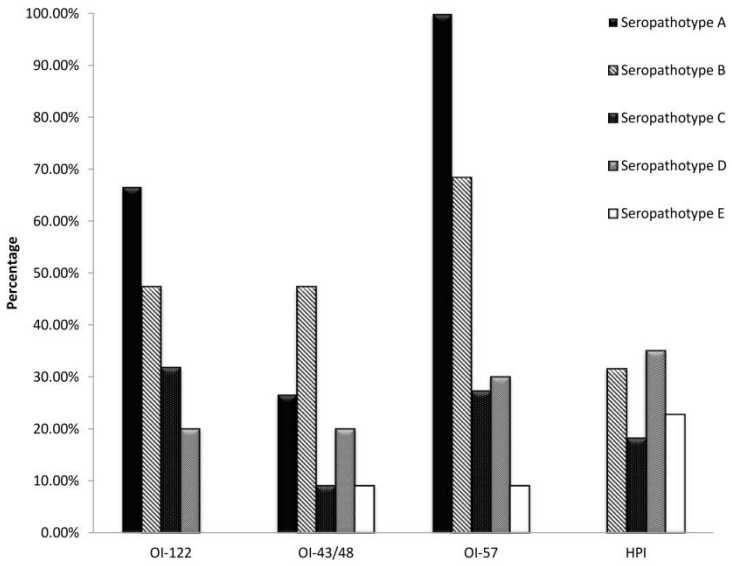


FIG III-2. Distribution of virulence genes located on OI-122, OI-43/48, OI-57 and high pathogenicity island (HPI) between *eae*-positive (n=54) and *eae*-negative STEC (n=44) strains.

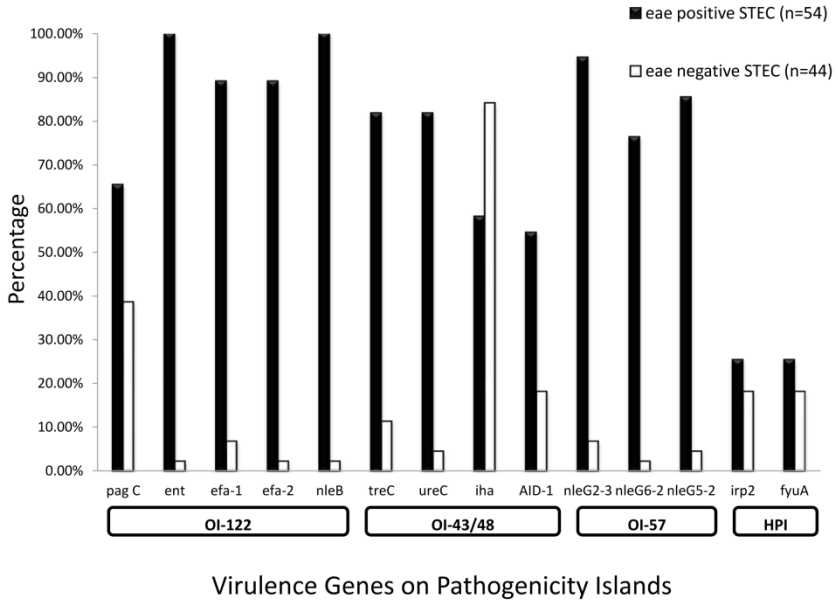


FIG III-3. Phylogenetic tree based on *iha* sequences from 67 *E. coli* and *Shigella* strains.

iha sequences were aligned and a tree was constructed using the maximum likelihood method with 2,000 iterations utilizing MEGA 5.05 (26). *iha* sequences from *eae*-positive and *eae*-negative STEC strains segregated into two distinct clades, clades I (with subgroup Ia and Ib) and II (with subgroups IIa and IIb). *iha* sequences from *eae*-negative STEC were marked in bold and italic, and *eae*-positive STEC strains were marked in bold. EPEC: enteropathogenic *E. coli*; EIEC: enteroinvasive *E. coli*; EAEC: eneteroaggregative *E. coli*; stx-producing EAEC: Shiga toxin-producing EAEC; ETEC: enterotoxigenic *E. coli*; UPEC: uropathogenic *E. coli*; NMEC: neonatal meningitis *E. coli;* and ABU: asymptomatic bacteriuria *E. coli.*

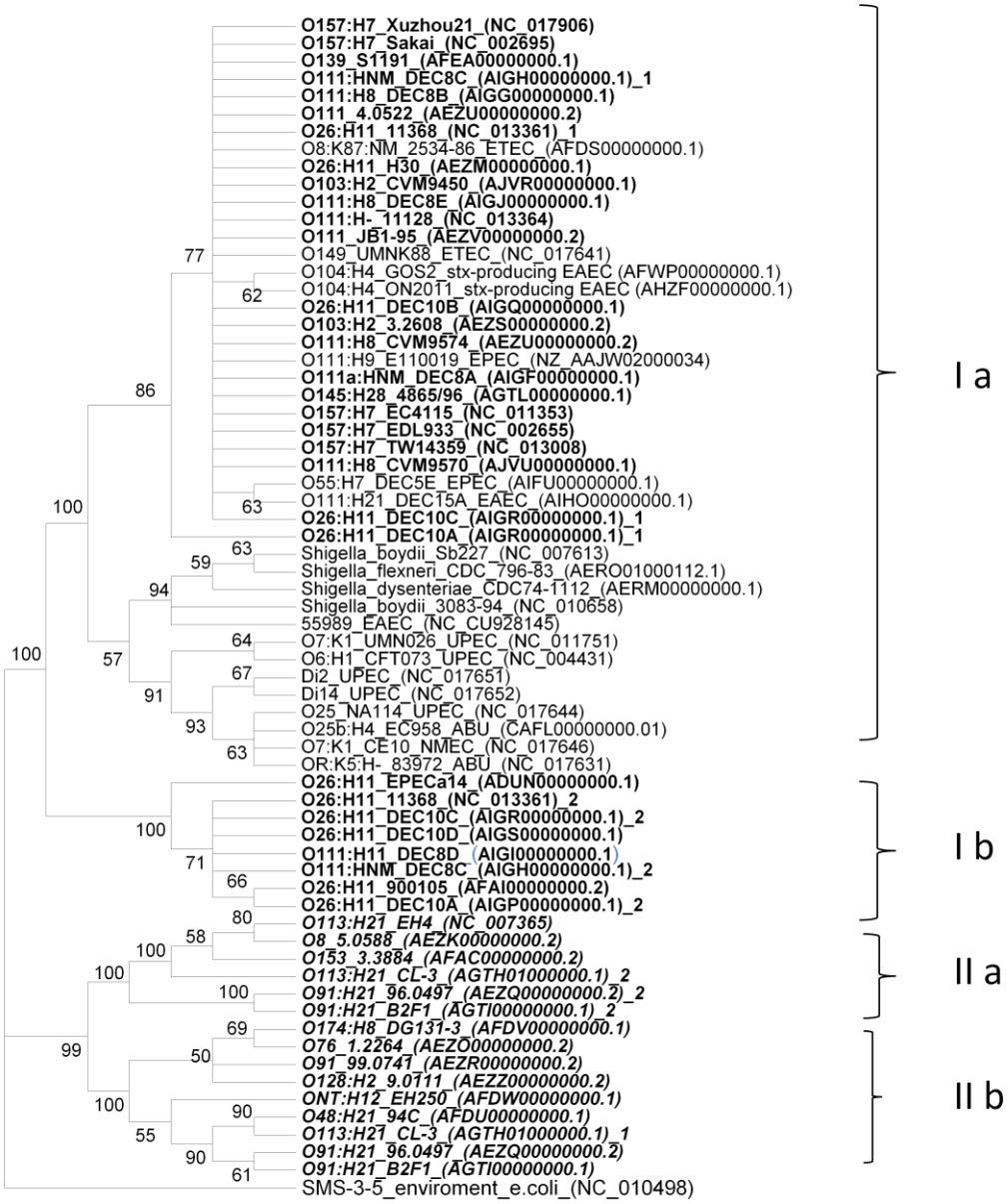
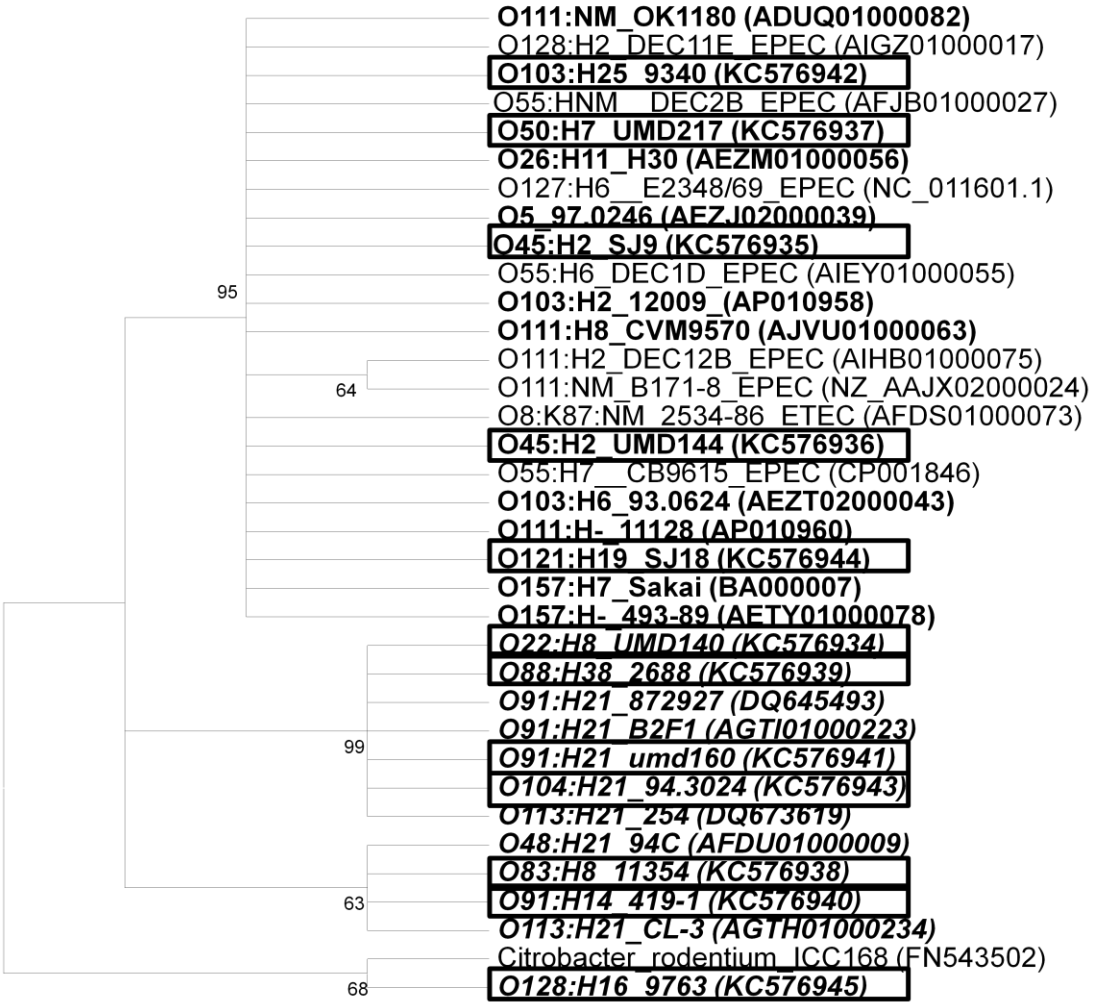

O157:H7_Xuzhou21_(NC_017906)
O157:H7_Sakai_(NC_002695)
O139_S1191_(AFEA00000000.1)
O111:HNM_DEC8C_(AIGH00000000.1)_1
O111:H8_DEC8B_(AIGG00000000.1)
O111_4.0522_(AEZU00000000.2)
O26:H11_11368_(NC_013361)_1
O8:K87:NM_2534-86_ETEC_(AFDS00000000.1)
O26:H11_H30_(AEZM00000000.1)
O103:H2_CVM9450_(AJVR00000000.1)
O111:H8_DEC8E_(AIGJ00000000.1)
O111:H-_11128_(NC_013364)
O111_JB1-95_(AEZV00000000.2)
O149_UMNK88_ETEC_(NC_017641)
O104:H4_GOS2_stx-producing EAEC (AFWP00000000.1)
O104:H4_ON2011_stx-producing EAEC (AHZF00000000.1)
O26:H11_DEC10B_(AIGQ00000000.1)
O103:H2_3.2608_(AEZS00000000.2)
O111:H8_CVM9574_(AEZU00000000.2)
O111:H9_E110019_EPEC_(NZ_AAJW02000034)
O111a:HNM_DEC8A_(AIGF00000000.1)
O145:H28_4865/96_(AGTL00000000.1)
O157:H7_EC4115_(NC_011353)
O157:H7_EDL933_(NC_002655)
O157:H7_TW14359_(NC_013008)
O111:H8_CVM9570_(AJVU00000000.1)
O55:H7_DEC5E_EPEC_(AIFU00000000.1)
O111:H21_DEC15A_EAEC_(AIHO00000000.1)
O26:H11_DEC10C_(AIGR00000000.1)_1
O26:H11_DEC10A_(AIGR00000000.1)_1
Shigella_boydii_Sb227_(NC_007613)
Shigella_flexneri_CDC_796-83_(AERO01000112.1)
Shigella_dysenteriae_CDC74-1112_(AERM00000000.1)
Shigella_boydii_3083-94_(NC_010658)
55989_EAEC_(NC_CU928145)
O7:K1_UMN026_UPEC_(NC_011751)
O6:H1_CFT073_UPEC_(NC_004431)
Di2_UPEC_(NC_017651)
Di14_UPEC_(NC_017652)
O25_NA114_UPEC_(NC_017644)
O25b:H4_EC958_ABU_(CAFL00000000.01)
O7:K1_CE10_NMEC_(NC_017646)
OR:K5:H-_83972_ABU_(NC_017631)
O26:H11_EPECa14_(ADUN00000000.1)
O26:H11_11368_(NC_013361)_2
O26:H11_DEC10C_(AIGR00000000.1)_2
O26:H11_DEC10D_(AIGS00000000.1)
O111:H11_DEC8D_(AIGI00000000.1)
O111:HNM_DEC8C_(AIGH00000000.1)_2
O26:H11_900105_(AFAI00000000.2)
O26:H11_DEC10A_(AIGP00000000.1)_2
O113:H21_EH4_(NC_007365)
O8_5.0588_(AEZK00000000.2)
O153_3.3884_(AFAC00000000.2)
O113:H21_CL-3_(AGTH01000000.1)_2
O91:H21_96.0497_(AEZQ00000000.2)_2
O91:H21_B2F1_(AGTI00000000.1)_2
O174:H8_DG131-3_(AFDV00000000.1)
O76_1.2264_(AEZO00000000.2)
O91_99.0741_(AEZR00000000.2)
O128:H2_9.0711_(AEZZ00000000.2)
ONT:H12_EH250_(AFDW00000000.1)
O48:H21_94C_(AFDU00000000.1)
O113:H21_CL-3_(AGTH01000000.1)_1
O91:H21_96.0497_(AEZQ00000000.2)
O91:H21_B2F1_(AGTI00000000.1)
SMS-3-5_enviroment_e.coli_(NC_010498)
I a
I b
II a
II b

FIG III-4. Phylogenetic tree based on *pagC* sequences from 34 pathogenic *E. coli* strains.

pagC sequences were aligned and a tree was constructed using the maximum likelihood method with 2,000 iterations utilizing MEGA 5.05 (26). *pagC* sequences from *eae*-negative STEC were marked in bold and italic, and *eae*-positive STEC strains were marked in bold. *pagC* genes sequenced by this study were marked by black frames. EPEC: enteropathogenic *E. coli*, ETEC: enterotoxigenic *E. coli*.



CHAPTER IV: PHYLOGENETIC ANALYSIS OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* BY WHOLE GENOME SEQUENCING

Abstract

Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) are emerging foodborne pathogens causing life threatening diseases and foodborne outbreaks. A better understanding of their evolution provides a framework for developing tools to control food safety. We obtained 15 genomes of non-O157 STEC strains, including O26, O111 and O103. Phylogenetic trees revealed close relationship between O26:H11 and O111:H11 and a scattered distribution of O111. We hypothesize that some STEC serotypes with same H antigens might share common ancestors.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are deadly pathogens, causing hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (1, 2). There are two categories of surface antigens (O somatic and H flagella), whose combinations are used to classify *E. coli*. O157:H7 has caused more outbreaks and HUS cases in the United States than any other STEC serotypes. However, there is a growing concern about the health risk of non-O157 STEC (3) as more than 470 serotypes of STEC are associated with human diseases (4). In the United States, non-O157 STEC cause an estimated 112,752 cases of illness each year, which is more than the number of cases (estimated 63,153) caused by *E. coli* O157:H7 (5).

Worldwide, six serogroups including O26, O45, O103, O111, O121 and O145 are most commonly isolated from human patients. Among the non-O157 STEC, serogroups O26, O111 and O103 are considered the most clinically important and frequently identified non-O157 STEC in severe diseases and foodborne outbreaks (6-8).

As emerging foodborne pathogens, the phylogentic relationship of STEC is not well understood. In this study, we used whole genome sequencing data to examine phylogenetic relationship of non-O157 STEC strains for a better understanding of the evolution history of these emerging pathogens.

Materials and Methods

Bacteria Strains. Fifteen STEC strains representing different PFGE patterns, isolation years, hosts, and *stx* gene profiles, including O26:H11 (5), O111:H11 (3), five O111:H8 (5), O103:H2 (1), and O103:H25 (1) were used in this study (Table 1). In addition, 28 *E. coli* genomes downloaded from the GenBank were included for phylogenetic study.

Pulsed field gel electrophoresis (PFGE). PFGE with *XbaI* was performed following a non-O157 PulseNet protocol (http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/5%201_5%202_5%204_PNetStand_Ecoli_with_Sflexneri.pdf). *Salmonella* Braenderup H9812 was used as control for PFGE. All the PFGE gel pictures were analyzed using BioNumerics software (Applied Maths, Austin, TX). Unweighted pair group means with arithmetic averages (UPGMA) was used to construct a dendrogram with a 1.5% band position tolerance.

***eae* subtyping**. *eae* subtypes were determined using PCR-RFLP as described by Tramuta et al (9). The following strains were used as positive controls: EDL933 (gamma), TW06584 (kappa), E2348-69 (alpha), TW07920 (epsilon), RDEC-1 (beta), TW10366 (rho), TW0350 (iota), TW07892 (eta), and TW01387 (theta). *E. coli* K12 was used as negative control.

Multiplelocus sequence typing. Seven housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*) extracted from genomes were selected for multilocus sequence typing (MLST) analysis as previously described for pathogenic

E. coli (http://www.shigatox.net/ecmlst/protocols/index.html). The MLST analysis was performed using MEGA 5.05 (10) with 2,000 iterations (model: maximum composite likelihood; substitution: transitions + transversions; Gamma).

Whole genome sequencing. The whole genome DNA of selected STEC strains were extracted with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Roche 454 GS-FLX+ system (Roche, Branford, CT) was used to perform DNA shotgun sequencing to obtain ~ 20 × coverage of draft genomes. The Roche Newbler (v 2.6) software package was used to conduct *de novo* assemblies. Multiple sequence alignment of all 43 genomes was performed using MAUVE (11). SNPs was extracted from the multiple sequence alignment and concatenated into pseudosequences by MAUVE. To further explore evolutionary relatedness, a parsimony phylogenetic tree based on whole genome wide SNPs was performed with 10,000 iterations by TNT (Tree analysis using New Technology) (12).

Results

Pulsed field gel electrophoresis and *eae* subtyping. The 15 STEC strains were grouped into two main clusters (FIG IV-1) that separated H11 strains (O111:H11 and O26:H11) from H8 strains (O111:H8). However, PFGE was not able to differentiate O111:H11 and O26:H11. In addition, the O26:H11 and O111:H11 strains shared the same *eae* subtype (*ß*), while the O111:H8 strains contained . It appeared that O111:H11 and O26:H11 were more closely related to each other than either was to O111:H8, according to PFGE profiles and virulence genes associated elements in the genomes.

Multi-locus sequence typing. In the MLST phylogenetic dendrogram, all the O111:H11, O26:H11 and O111:H8 strains formed one branch, with O26:H11 and O111:H11 clustering together in a lineage sister to the O111:H8 strains (FIG IV-2). It is interesting that O26:H11 strain DEC10B clustered with the O111:H11 strains, which also indicates the close relatedness between O26:H11 and O111:H11. Furthermore, five strains (12009, CVM9450, E22, DEC12C, and 03-EB-705) sharing the H2 antigen clustered together regardless of O serotypes. Genomic analysis revealed that O111:NM strain DEC12C carried *fliC* for the H2 gene.

Whole genome wide single nucleotide polymorphisms (SNPs) phylogenetic tree. The genome sizes of the 15 STEC strains ranged from 5.26 Mbp to 6.01 Mbp (Table IV-1). In order to analyze the phylogenetic relationship between non-O157 STEC, 28 *E. coli* genomes of various pathogenic *E. coli* including STEC, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), eneteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and Shiga toxin-producing EAEC

O104:H4 (from a German outbreak in 2011) downloaded from the GenBank were included for phylogenetic study. Multiple sequence alignment of all 43 genomes was performed using MAUVE (11) and approximately 183,470 single nucleotide polymorphisms (SNPs) were identified. Similarly as data shown by PFGE and MLST, the phylogenetic tree demonstrated that O26:H11 strains belonged to the same clade as the O111:H11 and O111:H8 strains, but grouped more closely with strains of the same H type (O111:H11) (FIG IV-3). All O111 strains but O111:H2 formed one clade (FIG IV-3), including O111 EPEC, O111:H8 and O111:H11. Additionally, we reconstructed maximum likelihood (ML) tree by Garli-2.0 (13) and Bio Neighbor Joining (BioNJ) (14) tree using SeaView 4 (15) (data not shown), displaying similar phylogenetic relationship with TNT tree with minor differences. For example, in the BioNJ tree, *E. coli* O26:H11 DEC10B grouped with the O26:H11 strains. The H2 strains including O103:H2, O45:H2 and O111:H2 strains were closely clustered together in all phylogenetic trees, including TNT, ML and BioNJ trees as shown in MLST dendrogram. The phylogenetic trees also supported that STEC O113:H21 and O91:H21 were closely related (FIG IV-3).

Discussion

Shiga toxin-producing *E. coli* are among the most important emerging foodborne pathogens. Recently, more and more outbreaks and sporadic disease are related to diverse serotypes of STEC. Among all the non-O157 STEC, O26, O103 and O111 cause most known outbreaks and HUS diseases. In this study, 15 STEC of O26, O103 and O111 from human and animals were sequenced. The phylogenetic trees were constructed to determine the evolutionary relationship of non-O157 STEC.

In this study, MLST, PFGE and whole genome wide SNP trees indicating that the H11 strains, including O26:H11 and O111:H11 (Figure IV-2 and IV-3) shared common ancestor; the H2 strains with different O antigens, namely, H2 group in Figure 3, shared common ancestor as well. Previous studies also have suggested that STEC strains with the same H antigens might share common ancestors. For example, Iguchi et al. (16) indicated that a close relatedness between STEC O103:H11 and O26:H11 by MLST analysis, which shared the same *eae* subtype (*ß-eae*) that was also inserted at the same t-RNA locus (*pheU*-tRNA). The O111:H11 strains used in this study also carry *ß-eae* as well (Figure 1). In addition, Konczy et al. (17) and Ziebell et al. (18) demonstrated that STEC O69:H11 was found closely related to O26:H11. Additionally, Konczy et al. (17) reported that H25 STEC strains (O103:H25, O119:H25, and O98:H25) and H21 STEC strains (O91:H21, O113:H21, O146:H21 and ONT:H21) were clustered together, separately, based on MLST. These data and our findings provided strong evidence that some STEC strains with common H antigens appear to originate from common ancestors. It is interesting that the four H groups included the following serotypes, O26:H11, O111:H11, O111:NM, O111:H2,

O103:H2, O103:H25, O45:H2, O91:H21 and O113:H21, which have been identified among the most important non-O157 STEC associated with outbreaks and HUS. Thus, it is possible that some most clinical and epidemic STEC serotypes with same H antigens might have evolved from common ancestors, respectively. It is possible that ancestral strains of those H groups share similar or same genetic background and/or environmental niche that could facilitate acquisition of *stx* and other virulence genes essential to STEC pathogenesis.

Our phylogenetic analyses demonstrated that the H groups were monophyletic while the serogroups were polyphyletic (FIG IV-3). Scattered distribution of different O111 strains suggested that strains from individual lineages might have acquired surface antigen genes independently in an ongoing parallel evolutionary process, such as *E. coli* O111:H21 DEC15E (O111 EPEC group), *E. coli* O111:H2 E22 (H2 group), *E. coli* O111:H8 CVM9634 (O111:H8 group) and *E. coli* O111:H11 CVM9545 (O111:H11 group) (Figure 3). Iguchi et al. (16) suggested that STEC O103:H2, O103:H11 and O103:H25 formed three different linages by MLST analysis and had distinct *eae* subtypes. The MLST and SNP phylogenetic trees in this study also supported that O103:H2 and O103:H25 were located at different linages (FIG IV-2 and FIG IV-3). Thus, just using serogroups may cause misleading conclusion about phylogenetic relatedness and health risk of STEC.

Pairwise distance matrix analysis with 2,000 bootstrap iterations (substitution: transitions + transversions; complete-delete option) (TABLE IV-2) were conducted to determine number of SNPs differences (standard deviation) between different selected groups using MEGA 5.05 (10), including H7, *eae*-types, H2, O26:H11 and

O111 groups. The values of base differences per sequence from averaging over all sequence pairs between groups were shown. The smallest distance value was found between O111:H11 and O26:H11 strains, confirming their close relatedness. Previous studies indicated that O157:H7 evolved from O55:H7 in a series of steps acquiring O157 antigen gene cluster and other virulence genes through HGT (19-22). The distance between O157:H7 Sakai and O55:H7 CB9615 was 4215 SNPs with a standard deviation of 37. Because O26:H11 strains located in the O111 clade in phylogenetic trees and display closer relationship with O111:H11 than O111:H8, it is possible that O26:H11 evolved similarly from an ancestral O111:H11 by antigenic shift from O111 to O26 (distance between O26:H11 and O111:H11 groups was 3617, Table 2). Sharing the same niche with other O26 strains may facilitate this genetic exchange. Comparative genomics analysis of O26:H11, O111:H11 and other STEC is underway to reveal the possible mechanism.

In conclusion, analyses based on whole genome wide SNPs, MLST, and PFGE suggest that in some occasions O serogroups appear not to track evolutionary relatedness among pathogenic *E. coli*. Instead, H antigens may be better markers of shared ancestry for some STEC serotypes.

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TABLE IV-1. Serotypes, pathotypes, toxin genotypes, sources and genome sizes of *Escherichia coli* strains used in this study*.

Strain	Serotype	Pathotype§	*stx*	Source	Size (Mbp)	Accession No.
CVM10021	O26:H11	STEC	*stx1*	cow	5.5	AKAZ00000000
CVM9942	O26:H11	STEC	*stx1*	cow	5.62	AJVW00000000
CVM10026	O26:H11	STEC	*stx1*	cow	5.57	AJVX00000000
CVM10030	O26:H11	STEC	*stx1*	cow	5.5	AKBA00000000
CVM9952	O26:H11	STEC	*stx1*	pig	5.5	AKBC00000000
CVM9634	O111:H8	STEC	*stx1+stx2*	cow	5.78	AKAW00000000
CVM9602	O111:H8	STEC	*stx1*	human	5.1	AKAV00000000
CVM9574	O111:H8	STEC	*stx1+stx2*	human	5.36	AJVV00000000
CVM9570	O111:H8	STEC	*stx1+stx2*	cow	5.51	AJVU00000000
CVM9545	O111:H11	STEC	*stx1*	cow	5.61	AJVT00000000
CVM9455	O111:H11	STEC	*stx2*	unknown	6.01	AKAX00000000
CVM9534	O111:H11	STEC	*stx1*	cow	5.46	AJVS00000000
CVM9553	O111:H11	STEC	*stx1*	cow	5.6	AKAY00000000
CVM9340	O103:H25	STEC	*stx1*	human	5.26	AJVQ00000000
CVM9450	O103:H2	STEC	*stx1*	human	5.39	AJVR00000000
CFT073	O6:K2:H1	UPEC	-	unknown	5.23	AE014075
SAKAI	O157:H7	STEC	*stx1+stx2*	human	5.59	BA000007
CB9615	O55:H7	EPEC	-	human	5.39	NC_013941
4865/96	O145:H28	STEC	*stx2*	human	5.23	AGTL00000000
53638	O144:?	EIEC	-	unknown	5.07	AAKB00000000
101-1	O-:H10	EAEC	-	human	4.98	AAMK00000000
MG1655	Unknown	Commensal	-	unknown	4.64	NC_000913
5.0959	H121:H19	STEC	*stx2*	unknown	5.37	AEZX00000000

TY-2482	O104:H4	EAEC+STEC	*stx2*	human	5.29	AFOG00000000
CL-3	O113:H21	STEC	*stx2*	human	5.05	AGTH00000000
B2F1	O91:H21	STEC	*stx2*	human	5.01	AGTI00000000
E24377A	O139:H28	ETEC	-	unknown	4.97	NC_009801
DEC12B	O111:H2	STEC	*stx2*	human	5.49	AIHB00000000
DEC12C	O111:NM	STEC	*stx2*	human	5.45	AIHC00000000
E22	O103:H2	EPEC	-	unknown	5.53	AAJV00000000
03-EN-705	O45:H2	STEC	*stx1*	human	5.3	AGTK00000000
12009	O103:H2	STEC	*stx1+stx2*	human	5.45	NC_013353
E110019	O111:H9	EPEC	-	human	5.38	AAJW00000000
DEC15A	O111:H21	EPEC	-	human	5.25	AIHO00000000
DEC15E	O111:H21	EPEC	-	human	5.23	AIHS00000000
DEC8E	O111:H8	STEC	*stx1*	human	5.32	AIGJ00000000
DEC8B	O111:H8	STEC	*stx1+stx2*	human	5.37	AIGG00000000
11128	O111:H-	STEC	*stx1+stx2*	human	5.37	NC_013364
DEC8D	O111:H11	DEC	-	human	5.46	AIGI00000000
DEC8C	O111:H11	STEC	*stx1*	cow	5.91	AIGH00000000
DEC10B	O26:H11	STEC	*stx1*	human	5.58	AIGQ00000000
EPECa14	O26:H11	STEC	*stx1*	unknown	5.44	ADUN00000000
11368	O26:H11	STEC	*stx1*	human	5.69	NC_013361

* Data on strains named with CVM were from this study; the rest were from GenBank. [§]STEC: Shiga toxin-producing *Escherichia coli* ; EPEC: enteropathogenic *Escherichia coli* ; EIEC: enteroinvasive *Escherichia coli* ; ETEC: enterotoxigenic *Escherichia coli* ; EAEC: eneteroaggregative *Escherichia coli* ; DEC: diarrheagenic *Escherichia coli* .

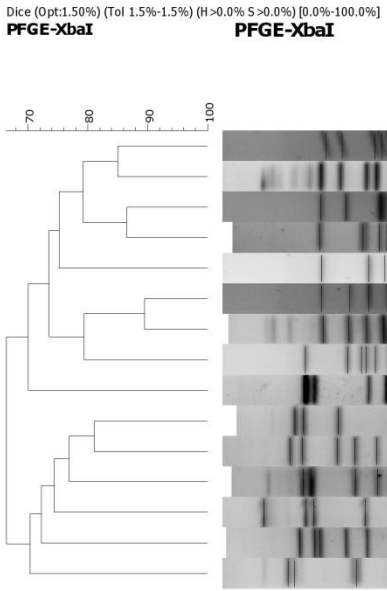
TABLE IV-2. Pairwise distance matrix analysis of six selected groups.

Group*	H7	*eae* negative	H2	O111 EPEC	O111:H8	O111:H11
H7						
eae negative	57246 (107)					
H2	58029 (139)	21523 (77)				
O111 EPEC	59530 (105)	23107 (98)	23442 (113)			
O111:H8	59498 (126)	23993 (75)	22558 (97)	21427 (83)		
O111:H11	59417 (113)	24157 (98)	22717 (123)	21512 (84)	4324 (35)	
O26:H11	57176 (108)	21913 (78)	22138 (107)	24045 (102)	6556 (42)	3617 (37)

***groups as by FIG IV-3**

Figure IV-1. Dendrogram of PFGE profiles of 15 O26, O103 and O111 STEC isolates.

Similarity of the PFGE profiles was based on Dice algorithm with 1.5% tolerance. O26:H11 and O111:H11 strains showed close relationship and grouped in the same cluster, and shared the same *eae* subtype.



CVM No	Source	Serotype	*stx*	*hlyA*	*eae* subtype
10030	Cow	O26:H11	*stx1*	+	beta
9952	Pig	O26:H11	*stx1*	-	beta
9534	Cow	O111:H11	*stx1*	+	beta
9545	Cow	O111:H11	*stx1*	+	beta
10021	Cow	O26:H11	*stx1*	+	beta
10026	Cow	O26:H11	*stx1*	+	beta
9553	Cow	O111:H11	*stx1*	+	beta
9942	Cow	O26:H11	*stx1*	+	beta
9450	Human	O103:H2	*stx1*	+	epsilon1
9574	Human	O111:H8	*stx1+stx2*	+	gamma2/theta
9634	Cow	O111:H8	*stx1+stx2*	-	gamma2/theta
9570	Cow	O111:H8	*stx1+stx2*	+	gamma2/theta
9602	Human	O111:H8	*stx1*	-	gamma2/theta
9455	Unkown	O111:H8	*stx2*	+	gamma2/theta
9340	Human	O103:H25	*stx1*	+	gamma2/theta

Figure IV-2. Dendrogram of MLST analyses using *aspC, clpX, fadD, icdA, lysP, mdh,* and *uidA*.

Strains 4865/96 (O145:H28), 101-1(O-:H10), 5.0959 (O121:H19), DEC12B (O111:H2) and E110019 (O111:H9) were not included in MLST study because at least one of selected gene alleles were either absent or only partially present.

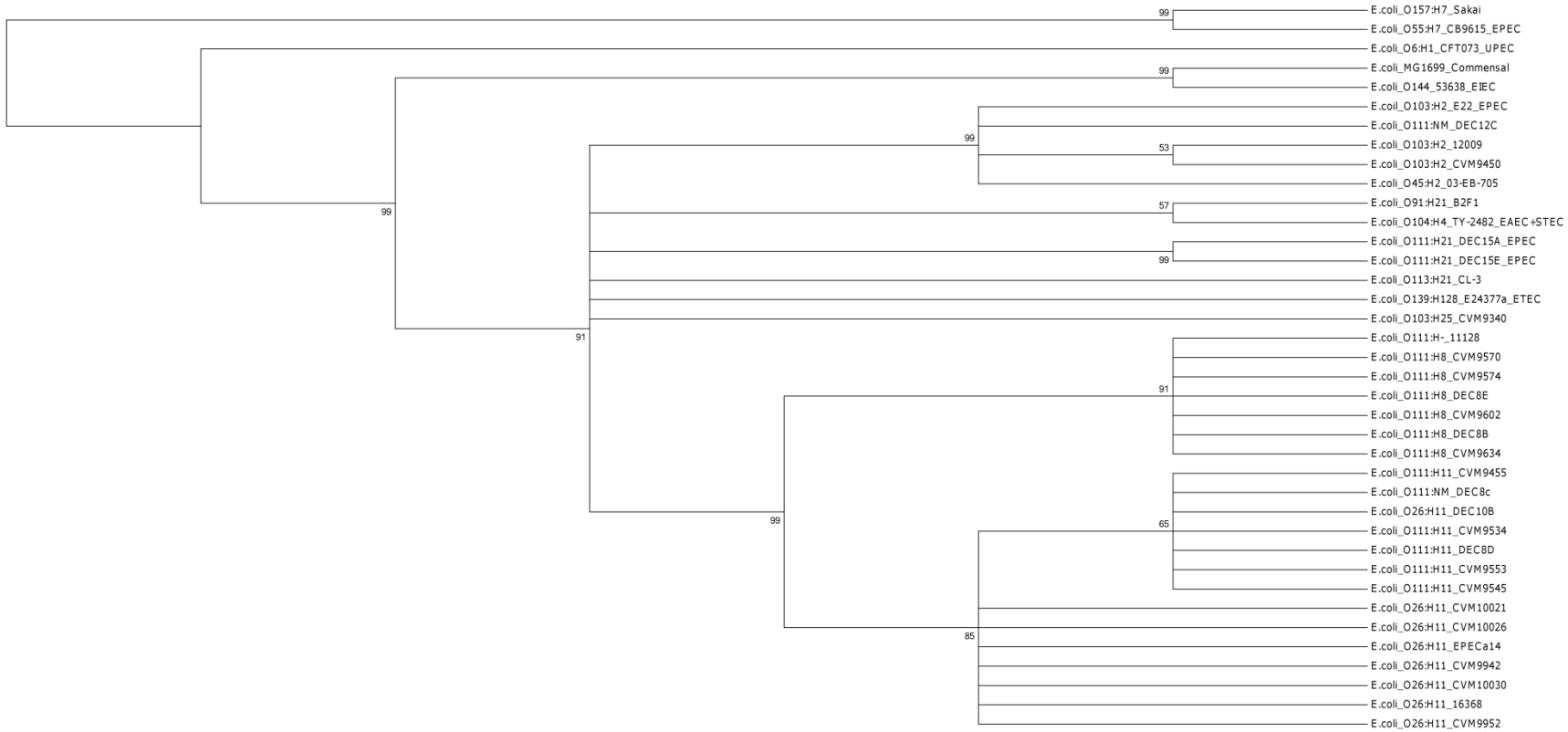
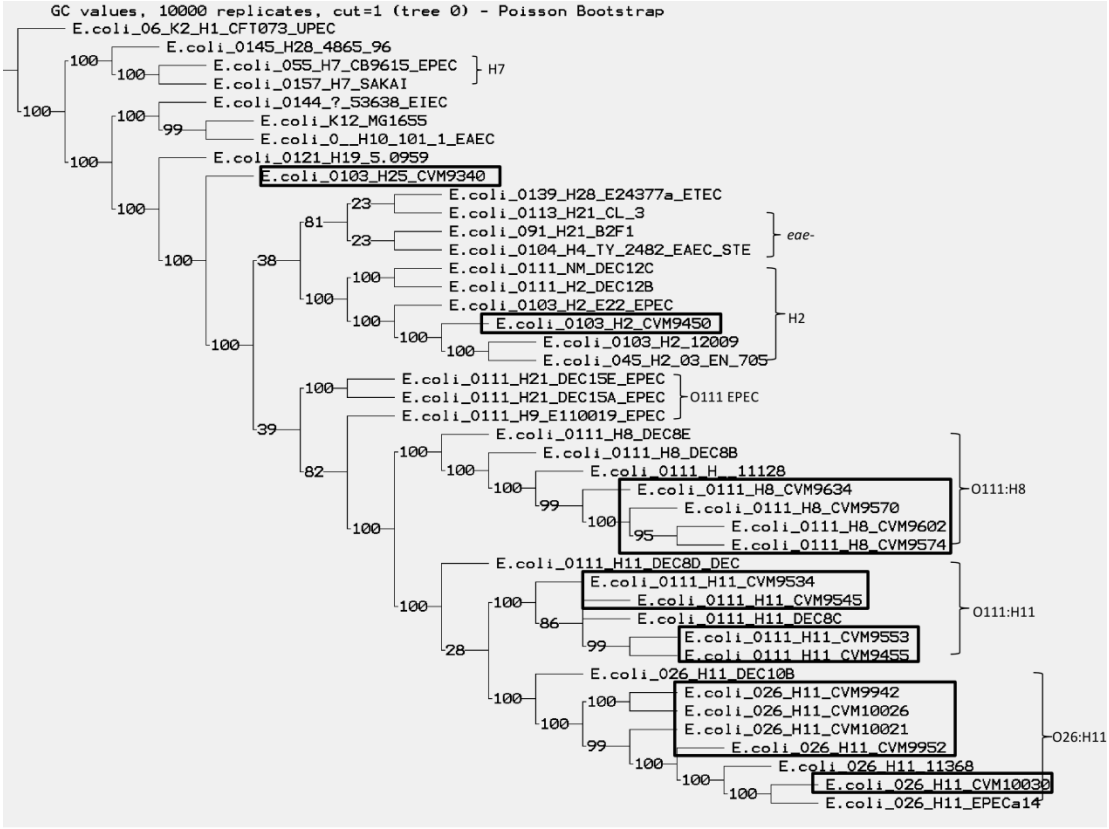


Figure IV-3. Parsimony phylogenetic tree of 43 *E. coli* from diverse pathotypes based on genome wide single nucleotide polymorphisms (SNPs) with 10,000 iterations.

Strains sequenced in this study were marked within frame. A total of seven subgroups were labeled, as shown in pairwise distance matrix (Table IV-2).



CHAPTER V: SUMMARY AND FUTURE STUDY

Shiga toxin–producing *Escherichia coli* (STEC) are emerging foodborne pathogens. There are at least 470 serotypes that have been reported associated with human illness. In the United States and worldwide, the report of incidences of non-O157 infections and foodborne outbreaks are continuously increasing. However, non-O157 STEC are generally not well studied. The objective of this study was to determine the prevalence of non-O157 STEC in retail meat and explore its pathogenicity islands distribution and evolutional relationship.

In chapter II, the prevalence and characterization of non-O157 STEC from retail meat were determined. Meat products, especially from cattle, can serve as vehicles in transmitting STEC to humans. Although the presence and characterization of O157 STEC has been very well studied, limited information is available about non-O157 STEC contamination in retail meat in the United States. Therefore, the first objective of this study was to determine the prevalence and characterization of non-O157 STEC from retail meats. Since no standard non-O157 STEC isolation method was available when this study started, a combination of a modified USDA MLG method was used to isolate non-O157 STEC from retail ground beef and pork. In total, 13 out of 271 beef and 12 out of 231 pork samples were positive for non-O157 STEC. Thirty-two non-O157 STEC isolates were recovered from beef (16) and pork (16). Although none of 32 non-O157 isolates were positive for *eae*, 12 were positive for *stx2dact*, which is one of most prevalent *stx* subtypes in *eae*-negative STEC strains associated with severe disease. In addition, eight isolates from pork and one

from beef were identified as O91 by PCR. O91 is a one of most commonly isolated serogroups from clinical samples. In addition, cytotoxicity assay demonstrated that 26 (81.3%) isolates produce Shiga toxin(s) that can cause cell damage to Vero cells. Besides, 17 (53.1%) isolates could resist two or more antimicrobials, which suggested that antimicrobial resistance is common among non-O157 STEC from retail meat.

In chapter III, distribution of pathogenicity islands (PAIs) was characterized in non-O157 STEC. Although the *stx* gene is the primary virulence factor of STEC, however, it was not a suitable marker to distinguish highly pathogenic with nonpathogenic or low pathogenic strains. Instead, pathogenicity island (PAI), which is normally highly prevalent in high pathogenic strains but almost absent in non-pathogenic strains of the same or closely related species, may be used as a marker to distinguish non-O157 STEC linked to severe disease with nonpathogenic or low pathogenic strains. In addition, PAI can also be used as a signature to identify new and emerging pathogenic STEC. Although some of other virulence genes have been identified associated with outbreaks and severe disease, the clear distribution of pathogenicity islands in non-O157 STEC and its association with diseases and outbreaks is still missing. In chapter III, 98 STEC strains were classified into seropathotypes A to E based on the association of individual serotype with diarrhea, severe diseases and outbreaks. PCR-RFLP was used to determine the *eae* and *stx* subtypes. Fourteen PCR targeting virulence genes located at different regions of an individual PAI were used to determine the presence of PAIs. OI-122 and OI-57 were identified as PAIs more prevalent in seropathotypes (SPT A, B and C) associated with severe disease than in other seropathotypes (SPT D and E). In addition, *ureC* and

terC, virulence genes located at OI-43/48, were also identified as statistically more prevalent in SPT A, B and C (associated with severe diseases). However, the high pathogenicity island, crucial for pathogenesis in *Yersinia*, was missing in SPT A (O157) and distributed evenly in SPT B, C, D and E. In summary, all the statistical comparisons indicated that OI-122, OI-57 and OI-43/48 might contribute to the non-O157 pathogenesis process and may be used as markers to predict highly pathogenic non-O157 in the future.

In addition, the association of OI-122, OI-57, OI-43/48 and HPI with LEE was analyzed in chapter III. Statistical analysis demonstrated that almost all virulence genes of OI-122, OI-57 and OI-43/48 were highly associated with *eae*, the marker gene for the presence of LEE. Although *iha* and *pagC* were highly prevalent in both *eae*-positive and *eae*-negative STEC, phylogenetic demonstrated that the two genes were separated with each other between *eae*-positive and *eae*-negative STEC, indicating that they may have different origins or have been separated a long time ago. In summary, both statistical analysis and phylogenetic studies indicated that *eae*-positive and *eae*-negative STEC may have huge differences in their pathogenic mechanisms. Additionally, the association of PAIs (OI-122, OI-57, OI-43/48 and LEE) with *eae*-positive STEC but almost missing in *eae*-negative STEC suggested that a complete different marker gene may need to conduct molecular risk assessment for *eae*-negative STEC in the future.

Besides LEE, OI-122, OI-57 and OI-43/48, there are seven other potential pathogenicity islands that were identified based on genomic and bioinformatics study in O157:H7 EDL 933. For example, OI-7 carries genes encoding a macrophage toxin

and ClpB-like chaperone; OI-28 contains genes encoding a RTX-toxin-like exoprotein and transport system; OI-47 encodes genes that have potential function as an adhesion and polyketide or fatty-acid biosynthesis system; OI-84 carries genes encoding O antigen polysaccharide; OI-108 encodes the adhesion-like auto-transporter, and the ATP binding component of a transport system; OI-115 carries genes for a type III secretion system and secreted proteins similar to the *Salmonella-Shigella inv-spa* host cell invasion genes; and OI-138 carries genes that function as a fatty-acid biosynthesis system. Those islands may be used as additional markers to distinguish highly pathogenic STEC with others. Currently, the developing of next generation sequencing and the small size of bacteria makes whole genome sequencing a relative cheap study tool for bacteria. A future whole genome study to determine the distribution of these potential PAIs may help us clearly identify the association of PAIs with different non-O157 STEC and also determine new markers to distinguish highly pathogenic STEC with low pathogenic ones.

In addition, as indicated by this study, the distribution of pathogenicity islands is totally different between *eae*-positive and *eae*-negative STEC. Genomic studies, especially comparative genomics study between *eae*-positive and *eae*-negative STEC, can help to fully unveil the infection mechanism difference between *eae*-negative and *eae*-positive.

In chapter IV, the phylogenetic relationship of non-O157 was analyzed by PFGE, multi-locus sequence typing (MLST), and whole genome wide single nucleotide polymorphism (SNP). Until now, the detailed evolutional relationship,

especially for non-O157, has not been unveiled in STEC. Among non-O157 STEC, O26, O111 and O103 are the serogroups that caused most of the known outbreaks and identifying its phylogenetic relationship is important for understanding their evolution and non-O157 STEC pathogenesis. In this study, a total of 15 STEC strains representing isolation years, hosts, and *stx* gene profiles, including O111:H11, O111:H8, O26:H11, O103:H2, and O103:H25, were selected for whole genome sequencing analysis using a 454-pyrosequencing system to obtain draft genomes. In addition, 28 pathogenic *E. coli* genomes, 16 of which belong to STEC, were used to analyze the phylogenetic relationship of STEC. PFGE, MLST and the whole genome level SNP study indicated that O26:H11 were closely related with O111:H11 and may have a common ancestor. WGS SNP analysis also indicated that O45:H2 and O1O3:H2 may also evolve from a common ancestor.

Compared with O26:H11, O111:H11 was much less commonly associated with severe disease and foodborne outbreaks. In the future, a whole genome level comparative study could unveil the potential factors that contribute to O26:H11 as a STEC that can cause severe disease and foodborne outbreaks. A comparative genomic between O103:H2, O45:H2 and O111:H2 may also unveil the mechanism explaining why O103:H2 are much more associated with severe disease and foodborne outbreaks than the other two serotypes with the same H type.

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